

Conflicts of interest on the greenhouse

Far from being a diversion, last week's argument at Berlin over the obligations of poor countries on global warming is a sign that neglected issues are at last being taken up.

NOBODY should be surprised, let alone offended, that the most conspicuous row last week at the Berlin conference on climate change was the emergence of the long-simmering conflict of interest between developed and developing countries. Since long before the Rio conference in 1992, it has been clear that, if global warming is a real problem, it is strictly a global problem. Atmospheric concentrations of carbon dioxide and other greenhouse gases do not recognize frontiers. In due course, equity requires that the same rules should apply to all emitting countries. The difficulty is that the now-poor countries legitimately claim that their economic advancement should not in the short run be unreasonably impeded. The task ahead is to devise a framework for agreement on the progressive containment of greenhouse gases in which the obligations as well as the needs of the developing countries are made explicit. By the end this week, we shall know whether the political representatives of governments have agreed to face up to this issue or whether they have fudged it.

The technical issues are difficult enough. How can the emission of greenhouse gases be accurately contained within some limit yet to be determined if nobody knows for sure what the emissions are? That rhetorical question argues for comprehensive monitoring, to agreed standards of accuracy, of the industrial practices of developing as well as developed countries. And because the economic costs of the restrictions that may be necessary are huge, verification of national monitoring arrangements will be necessary, as in arms control agreements. Will China and India, two prodigious coal-burning countries now conveniently exempt from restrictions by their classification in "Annex II" of the Rio treaty, swallow that requirement?

The simple riposte, much in evidence last week, that it will be time enough to think of shouldering developing countries with obligations when their per capita consumption of fossil fuels is comparable with that of developed countries will not wash. If that state of affairs should come about, the present rate of carbon dioxide emissions would probably multiply by between five and ten.

Moreover, there are poor-country practices, from logging to the cultivation of rice in paddy-fields, that need to be taken into account. Even the use of fuelwood is not, as often held, environmentally benign; true, the practice merely returns to the atmosphere carbon dioxide that was there at an earlier stage, but that is irrelevant if the atmosphere already contains too much for comfort.

The inefficient use of traditional fossil fuels in developing countries is similarly unacceptable. That is not a matter of technology alone, but of economics as well. Rich countries will have to accept that there can be no single standard of efficiency for them and for their poor relations. Those who build and operate steam boilers, say, must forever balance the capital cost (and thus the efficiency) of their plant against the local cost of fuel, which may well be a function of local wage rates and so be less in poorer countries. But the continued operation of plant that is inefficient by local standards is both a needless local cost and a gratuitous source of carbon dioxide. If the threat of global warming requires a global limit on emissions, the operation of all locally inefficient plant is a route to global impoverishment that will, among other things, restrict the capacity of now-rich countries to provide their poor relations with the technical and financial assistance they will eventually need.

The social and political issues not yet tackled are even more obdurate. What, in a regime designed to head off global warming, is a country's 'entitlement' to emit greenhouse gases? Stabilizing carbon dioxide emissions from the rich countries at, say, 1990 levels will not yield steady state; stabilizing emissions from poor as well as rich countries at 1990 levels would eventually do so, but would inequitably prevent development. So why not a per capita standard instead? There is much to be said for such an approach, but that would act inequitably against countries (such as China) that had taken steps to control their population growth. Although it might be possible to hammer out compromises on these questions in, say, present circumstances, a durable framework for a protocol to head off global warming requires an understanding that does not have to be renegotiated whenever circumstances change. And the principles should be agreed before the details are fixed.

What the Berlin meeting shows is that these difficult questions have not so far been asked, let alone answered. That is why the best hope is that there will soon be another conference, aimed at tackling them. The danger is that these issues will be too difficult for the world community to face or that they will be so regarded. That will be to sell the pass. A global warming regime will be effective only if predicated on some degree of global government. Politicians may not like being pushed in that direction by what seems to be a strictly technical difficulty but it will not be the first time — remember nuclear weapons — that this conclusion has been inescapable. □



ESO objects to police 'harassment' in course of telescope dispute

London. A sharp dispute over the construction of the world's largest optical telescope on a mountain top in northern Chile entered a new phase last week when Chilean police forced their way into the local offices of the European Southern Observatory (ESO).

Javier Jimenez, a Chilean judge, accompanied by a contingent of local police, entered the ESO buildings in the town of Cerro Paranal, at the base of the mountain that includes the observatory site, at lunchtime on 30 March.

After what is described as a "tense" but incident-free 90-minute visit, the officials left the offices armed with photographs and a comprehensive inventory of all building work completed since a court ordered the initial suspension of telescope construction last spring (see *Nature* 378, 676; 1994).

ESO officials were told by Jimenez as he left that any work completed after the ban would be demolished, and that ESO's eight member states would have to cover the costs. "This situation is totally unprecedented," says Richard M. West, a spokesman for ESO, which is based in Munich, Germany. "In our opinion, this is a clear violation of international law."

In a statement, ESO said that the act of the Chilean court had raised "to a new level the quality of the harassment to its activities in Chile" and would result in "substantial financial damage" to the Very Large Telescope (VLT) project.

The Chilean courts last year ordered construction work on the DM500-million (US\$360 million) (VLT) to stop when a Chilean family, the Latorres, filed an appli-

cation claiming ancestral ownership of the VLT's proposed site, donated to ESO by Chile in 1987.

ESO opposed the suspension on the grounds that an intergovernmental agency should be immune from local laws and that the dispute over land ownership was between the Latorres and the Chilean government, rather than with ESO, which has agreed with the government to construct

intergovernmental accords — and suggest that this could damage the country's attempts to lure much-needed foreign investment. "This is a very serious matter," says West. "It means that an agreement with Chile is no guarantee that your property will be protected."

An extraordinary meeting of ESO's governing council has been called to discuss the Chilean situation. West says that when they meet, the council's 16 members — made up of one politician and one astronomer from each member state — will be prepared to discuss "all available options".

European Southern Observatory

These are widely thought to include the possibility of moving the observatory site out of Chile — and perhaps even abandoning the project altogether. The latter option, however, is not generally thought to be realistic, as 70 per cent of the project's budget has already been committed. The tele-

scopes are already in production, while the buildings are practically ready for use.

Meanwhile, Chile's own astronomers have mixed feelings about the telescope, disappointed that they have not been allocated more time on the facility when it is eventually completed. Monica Rubio, an astronomer at the University of Chile, complains that the land dispute has overshadowed the issue of time for Chilean astronomers, which is far more crucial for Chilean science.

Rubio and other astronomers say the allocation of 10 per cent of the telescope's operational time for Chilean astronomers has still not been guaranteed by the government. "We are not asking for anything special," she says. "In Hawaii [the site of several US and European telescopes], the quota is 15 per cent, while it is 20 per cent [for Spanish astronomers] in the Canary Islands."

Rubio says that if Chilean astronomers do not get the guarantee they are seeking, "the telescope might as well be in a different country; it will make no difference to Chile". Others claim that Chilean public opinion is remains mixed about the whole dispute, with many people arguing that the Latorres should be compensated if the land did indeed legally belong to them.

According to Rubio the difficulty for Chileans — as indeed it is for ESO — is that the issue is no longer about science. "It has, instead, become a symbol of the struggle between the political powers and the judiciary over decision-making in Chile," she says.

Ehsan Masood

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

Work in progress: construction of the Very Large Telescope on Mount Paranal remains the subject of controversy over land ownership.

the telescope by 1997. "We never agreed with the injunction to stop work," says West. "We are the unfortunate third party."

While the Latorres family has been pursuing its case in the courts — it is now before Chile's higher supreme court — ESO has stuck to its original construction timetable, in the belief that the Chilean government, under the terms of the agreement, will eventually grant immunity from any court decision going against the observatory.

Last week's police action, however, marks a turning point in the dispute, and could have wider implications. Some feel that it has laid Chile open to suggestions that the state has shown itself unable to honour

Takeda gains access to HGS data-bank

London. Takeda Chemical Industries Ltd., Japan's largest pharmaceutical company, last week signed an agreement with Britain's SmithKline Beecham (SB) giving it access to sequence data on the human genome which the British company has licensed under a \$120 million deal with Human Genome Sciences (HGS) Inc. of Rockville, Maryland.

According to Kunio Takeda, the president of the Japanese company which has recently been seeking to expand its efforts in basic research, the collaboration with SB and the access to HGS's gene sequence data base will allow it to "efficiently utilize genomic information for drug discovery."

Other companies are reported to have been reluctant to enter agreements with SB to use the HGS data because of the terms under which access is being offered (see *Nature*, 371, 463; 1994). But a statement from SmithKline says that, under the terms of a letter of intent signed with Takeda, the two companies will collaborate on research in "the discovery and development of novel pharmaceuticals based on genomic technology."

In addition to gaining access to sequencing data produced by HGS and The Institute for Genomic Research, the agreement will also allow the Japanese company to make use of bioinformatics technology developed by SB and HGS. □

NIH faces plans to privatize clinical centre

Washington. US health department officials are considering plans to privatize all or part of National Institutes of Health (NIH) Clinical Center, the centrepiece of the NIH intramural research programme.

The privatization plan, which would involve transferring responsibility for the centre's functions to private contractors, has originated in the office of Philip Lee, the assistant secretary for health. It is one of a number of proposals being considered by Donna Shalala, the secretary of health and human services.

Suggestions of different ways of reducing the number of employees are seen in Shalala's department as a contribution towards President Bill Clinton's "reinventing government" initiative. But the plan has caused widespread alarm at the NIH campus at Bethesda, Maryland, where the Clinical Center employs 2,000 of its own staff, and also houses a further 4,000 individuals from the various NIH institutes.

Helen Smits, the deputy administrator of the Health Care Financing Administration, who has been asked by Shalala to assess options for privatization at the Clinical Center, visited the campus last week to begin talks with representatives of management and staff.

According to John Gallin, director of the centre, Smits indicated after the first round of meetings that she did not favour privatization of the entire operation. "We were impressed by her flexibility," Gallin says.

At the meetings, Smits was told of the obstacles that will inevitably confront even a partial privatization of the Clinical Center. One is that attempts to contract out either of the two most obvious service functions at the centre — cleaning and catering — would leave the rest of the centre exposed to legal challenges over the under-representation of black employees.

The contracting out of work by laboratory technicians, on the other hand, could create different problems if the staff involved chose to exercise their right to stay in government service by moving from the Clinical Center to other parts of NIH, as in doing so they could displace more junior staff from their jobs.

NIH managers have been trying to persuade Smits that the centre could most effectively increase its efficiency in areas such as purchasing where, they suggested, government-run hospitals could cooperate in order to cut costs. Smits, who has two months to report back to Shalala, has set up a panel of experts from both inside and outside NIH in order to help her explore privatization options.

The plan to privatize services at the Clinical Center is separate from impending plans to replace the ageing, main clinical centre building with a new 250-bed hospital, at an

estimated cost of \$380 million. Gallin and other officials say that the rebuilding plan is still on track — even though there is no extra money in NIH budget projections to pay for it.

The institutes' budget for the 1996 financial year, now being considered by Congress, includes \$26 million for the design of the new hospital. The 1997 budget is expected to include provision for its construction, but it has not been determined which programmes should be cut to enable this. Gallin says the NIH is exploring "other approaches" to finding money for the new hospital, but that talk of private funding would be "speculation" at this stage.

The privatization proposal is part of a broad effort by the Clinton administration to cut staffing levels across the federal government. But NIH officials are worried that, in this case, the administration may proceed in order to meet its staffing targets — even if

no money is likely to be saved. A memorandum last week from Shalala to all department staff pointedly failed to deny that the privatization of the Clinical Center was under consideration.

The centre is a critical component of the NIH's \$10 billion biomedical research programme, accounting at any given time for 40 per cent of all hospital beds used for patients taking part in clinical research. Its supporters say the role of the centre will become even more critical in coming years, as financial pressure makes it harder for medical centres outside NIH to find beds for patients in clinical trials.

But the administration's proposal for radical change at the centre may be only the first of a series of major upheavals at NIH as, after years of strong growth, it comes to terms with a budget that is unlikely to grow fast enough to cover inflation.

Colin Macilwain

New 'security' role urged for science

Washington. Social collapse in the poor nations of the world and global environmental degradation are now the principal threats to US national security with which science must contend, Vice President Al Gore told a high-powered forum of scientists in Washington last week.

Gore described the use of science and technology to build better weapons to defend the US from external threats as an "entirely inadequate" response to national security problems. "We need to ask how science and technology can assist us in coping with new challenges which often feature a multifactorial assault on society," he said.

Gore was addressing the 400 science and technology experts invited to a meeting at the National Academy of Sciences to help the Clinton administration draft a new policy document on the role of science and technology in national security.

Like last summer's basic research policy document *Science in the National Interest* (see *Nature* 370, 401; 1994), the document is supposed to explain the principles for the administration's support of science.

Critics contend that such statements, intended for a broad audience and lacking specific proposals on the allocation of money, serve little useful purpose. But

Jane Wales, associate director of national security at the White House Office of Science and Technology Policy (OSTP) and organizer of the forum, said that both the meeting and the document would help OSTP to "determine if it was successfully supporting the President's national security strategy".

The policy document is likely to reflect the administration's broad view of national security, Wales says, embracing the need for sustainable economic development in developing countries and a response to environmental threats, as well as military preparedness.

This may, however, bring the administration into conflict with the Republican majority in Congress, which is sceptical about sustainable development and continues to equate national security with military strength.

Senator Sam Nunn (Democrat, Georgia) and William Perry, the defence secretary, used the forum to call for more support from US universities in trying to find new tasks for Russian scientists formerly engaged in nuclear weapons research. "We need every research university in the US to have at least one or two [collaborative projects] on board," said Nunn.

Charles Curtis, under-secretary at the Department of Energy, said science and technology could help in attempts to counter internal security threats, such as last month's chemical attack on the Tokyo underground railway system. But Joshua Lederberg, the Nobel prizewinning geneticist, warned that deploying technology to such ends would be difficult.

C.M.



Gore: seeking more support for policies.

Monitoring plan gets low marks, low budget

Washington. An ambitious attempt by the US Environmental Protection Agency (EPA) to develop a comprehensive, national 'report card' for the environment is being scaled down following continuing criticism from both inside and outside the agency.

Some fear that the criticism may help to make the programme a potential target later this year as Congress searches for government programmes to axe.

The Environmental Monitoring and Assessment Program (EMAP) began in 1990 with a challenging goal: to establish a nationwide monitoring system that would provide scientists and policy-makers with fundamental, long-term data on ecological change. But after spending \$135 million over five years, the project is still far from reaching its goal.

Now a National Research Council panel, set up in 1991 to review EMAP, has stated that it "continues to question whether and how well [the project's] goals can be achieved". The panel, chaired by Richard Fisher of Texas A&M University, will release next week its final report containing an overall assessment of the project.

Of EMAP's many problems, perhaps the most serious is that it has not yet identified which indicators it would use to measure ecological change across different kinds of ecosystems. This alone requires a great deal of research, according to the NRC panel. But EMAP has been slow to get started.

The panel also questions the proposed sampling methodology. It claims that this "may operate at too coarse a scale in space and time to detect meaningful changes in the condition of ecological resources", according to the group's final report.

Some of EMAP's woes are attributable to money. What was originally envisioned as a \$100 million per year programme has never



Still waiting: proposals for a nationwide grid of monitoring sites may be abandoned to cut costs.

even come close. The project's 1995 budget was only a third of that amount.

But the NRC panel also details numerous administrative problems, including a lack of permanent staff assigned to the project and insufficient scientific advice from outside EPA. The end result is that after five years, EMAP has yet to come into focus. "They were still spending an awful lot of money answering very few fuzzy questions," according to one panel member.

Even though programme managers say they are now implementing many of the recommendations in the NRC report, it may be a case of too little, too late. Robert Huggett, the new head of EPA's Office of Research and Development and himself an ecologist, has had his sights set on EMAP from the day he came to the agency.

Now, as he sets out to revamp EPA science — another NRC panel last month applauded these efforts — Huggett has called for EMAP to be drastically restructured and scaled down. The idea of a nationwide grid of monitoring sites has been abandoned. In its place, the project would merely establish a few sites to take more in-depth measurements, while developing a set of scientifically valid indicators of ecological health.

With Huggett's restructuring in mind, Congress has already moved to reduce the programme's budget. A Senate bill expected to be approved this week rescinds \$6 million of the \$37 million already allocated to EMAP for this year.

When members of the Senate and the House of Representatives confer later this month to reconcile their two versions of the 'rescission' bill, the money for EMAP is unlikely to be restored. "I haven't had a single member [of Congress] come into my office to ask that the money be put back," says one congressional staff member who works on EPA's budget.

The fact that the Senate Appropriations Committee singled out EMAP, a relatively obscure programme, as a target for 1995 cuts suggests that the 1996 budget hearings — the agency is requesting \$36 million for the programme — will be even rougher. Huggett will shortly have to convince a sceptical Congress that EMAP, or whatever reduced research programme replaces it, is worth saving.

Tony Reichhardt

MIT links biologists to engineers

Boston. The Massachusetts Institute of Technology (MIT) has set up a Center for Biomedical Engineering (CBE) intended to stimulate closer collaboration between research scientists, engineers and clinicians. In particular, the centre's programme is geared towards bringing engineering into closer contact with the most recent developments in cell and molecular biology.

"MIT has supported superb biomedical engineering work in the past, but the research was conducted by individuals scattered throughout the university," says the director of the new centre, Douglas Lauffenburger, himself a chemical engineer. "This work has never been coordinated through a single centre responsible for putting together multidisciplinary teams of biologists, chemists, and engineers."

The centre will focus on three general areas: molecular engineering, cell and tissue engineering and physiological systems. According to Lauffenburger, the centre is ideally situated for this kind of work, given the strengths in engineering, biology and medical research to be found at MIT and its Whitehead Institute, Harvard Medical

School and its affiliated hospitals, and other Boston institutions.

"It's hard to find another city in the world that is better placed in these three fields," he maintains. "There are many talented people here with a wide range of expertise. My job is to combine their skills in new ways with the eventual goal of improving health-care technology."

The centre was set up in January this year, but its new laboratory facilities will not be ready until the end of next year at the earliest. Nevertheless, many MIT faculty members from a variety of departments have already joined, and new research collaborations have formed as a result.

For example, Ian Hunter, a mechanical engineer at MIT who is an expert in small-scale robotic devices, has teamed up with Paul Matsudaira, a biologist at the Whitehead Institute, to develop new ways of studying the structural properties of proteins such as DNA. "Before the centre was created, neither was aware of the other's work," Lauffenburger says. "Ultimately, we hope to bring about interactions that have not even been considered previously." **Steve Nadis**

Indian institutes to raise transfer efforts

New Delhi. The Indian government has launched a Rs630 million (US\$20 million) scheme designed to help transfer knowledge from its leading academic institutions to the industrial arena.

The five Indian Institutes of Technology — in Madras, Delhi, Kharagpur, Kanpur and Bombay — as well as the Indian Institute of Science in Bangalore will each help to set up what is being described as a national mission for technology development and transfer.

In contrast to previous schemes, industry will be a major partner in the new programme, and will be expected to contribute 25 per cent of the costs of individual projects. **K.S. Jayaraman**

Science budget faces continuing squeeze in South Africa

Cape Town. South African scientists have been disappointed at the new science budget, which will be increased by only 1.8 per cent in the 1995-96 financial year, to a total of R834 million (US\$230 million). It had been hoped that the creation of a new ministry responsible for science might have prevented a further erosion of spending on science, which fell by 27 per cent between 1987 and last year.

The increase in the science vote is less than the average spending increases of just over seven per cent granted to government departments as a whole. The total budget increase is in line with the expected level of inflation; but a large proportion will be taken up servicing the national debt.

Despite his commitment to increasing expenditure on research and development, Ben Ngubane, the Minister of Arts, Culture, Science and Technology, has clearly failed to persuade his cabinet colleagues to boost funding this year. Nor has he been able to introduce any transparency into the process of allocating the science vote among the research councils.

As with the Scientific Advisory Council appointed by the previous National Party government, the recommendations of the minister's interim advisory committee were not made public. It is therefore unclear whether the differing increases awarded to the various councils reflect the views of this committee or those of the ministerial committee that makes the final allocations.

The biggest increases this year were awarded to the two largest councils, the Council for Scientific and Industrial Research and the Agricultural Research Council, which both carry out in-house research. They received increases of 5.9 per cent and 6.3 per cent respectively, effectively reversing the effects of last year's trend, when they received the smallest rises.

By contrast, the Foundation for Research and Development and the Medical Research Council, the two councils that distribute a significant proportion of their funds to the tertiary education sector, each received funding increases of only 2.7 per cent. The Council for Geosciences was given a budget increase of 4.5 per cent.

Michael Cherry

● President Nelson Mandela has dismissed his wife Winnie as deputy minister of Arts, Culture, Science and Technology, and replaced her in this post with Brigitte Mabandla, a Member of Parliament who represents the African National Congress. Mabandla is a prominent feminist and a former exile. She is a lawyer by training, and appears to be a popular choice to succeed Mrs Mandela.

Aerosols' role simulated in new global warming model

London. Scientists at the UK Meteorological Office's Hadley Centre claimed success last week in their efforts to develop a computer model which accurately simulates the way in which aerosols mitigate the process of global warming.

According to the scientists, preliminary results circulated to delegates attending the first full governmental meeting of signatories to the Climate Change Convention, signed in Rio de Janeiro in 1992, suggests that, for the first time, a general Circulation Model has been able to replicate in broad terms observed temperature changes over the past 130 years (see figure).

Previous studies by the International Panel on Climate Change (IPCC) showed that greenhouse gases acting on their own would have led to an increase in global temperature of between 0.6 and 1.3°C Centigrade over this period.

An experiment based on the new model, taking into account the emissions of natural

and man-made sulphates, produced a simulated temperature rise close to the observed value of 0.5°C.

"What we have done is reduce the uncertainty of the models," says David Bennetts of the Hadley Centre. "The important thing is that you can put the aerosols in, and the model appears to behave close to what is observed." A report circulated at Berlin by UK officials describes the experiment with the model, which took three months to run on supercomputers, as "an important step forward in climate prediction science."

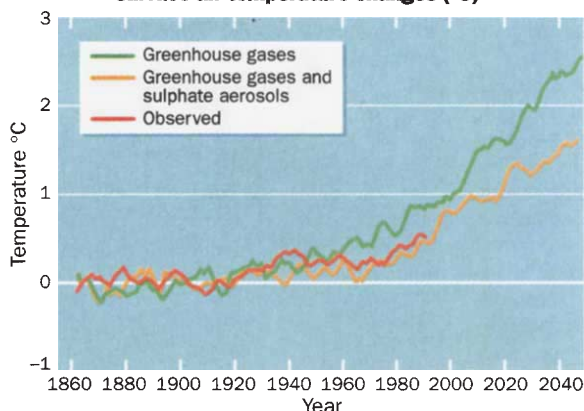
At the same time, they acknowledge that more work needs to be done on, for example, the fact that regional calculations with the model show a greater variability from observed temperature changes than the global model. "It is clear that the location and magnitude of specific changes are not replicated fully — as might be expected from this first attempt to allow for the influence of aerosols," the scientists state in last

week's report (a full paper describing the results of running the model has been submitted to *Nature*).

But the models already indicate that the role of aerosols in climate change may have important policy implications. For example, the fact that regional reductions in sulphate aerosol emissions can, according to the model, lead to local temperature increases is likely to cause a dilemma to governments faced with pressure to take just such action in order to reduce the dangers of acid rain.

David Dickson

Predicted (1860–2050) and observed (1860–present day) surface air temperature changes (°C)



Major ozone thinning found over Arctic

London. A joint European research project has detected new evidence of a significant reduction in ozone levels in the atmosphere, this time over the Arctic. But the researchers say they remain uncertain how much of the reduction is due to ozone depleting chemicals, rather than to dynamic changes caused by the mixing of air masses at different ozone levels.

Analysis of both ground and air-based observations by the Second European Stratospheric Arctic and Mid-latitude Experiment (SESAME) over the Arctic polar vortex — a moving body of cold air found mostly above northern Europe — has revealed a reduction of up to 50 per cent in ozone levels at altitudes between 16 and 18 kilometres.

"A large part of this difference is likely to be caused by the chemical destruction of ozone," the SESAME team said in a statement last week. "But further analysis is required to quantify and confirm this."

SESAME is a two-year experiment funded by the European Union to look at the causes of ozone depletion in the northern hemisphere. The researchers found ozone levels in parts of the polar vortex falling at about 0.7 per cent a day. They point out that the amount of ozone over middle latitudes in the northern hemisphere has declined by more than seven per cent over the last 15 years.

SESAME's measurement phase is now nearing completion. It will be followed by a period of intensive analysis.

E. M.

International fund fosters cross-border Irish research

Munich. Neatly timed to demonstrate the general benefits of the Anglo-Irish peace initiative, three major cross-border research programmes have just been launched to promote collaboration between scientists from the north and south of Ireland, who have tended to be isolated from each other.

The programmes are financed by the International Fund for Ireland (IFI), set up by the Irish and British governments under the Anglo-Irish peace agreement of 1986 to promote political stability by boosting the economies on both sides of the border.

The fund distributes a total of £20 million (US\$32) a year, provided by the United States, Canada, New Zealand and the European Union (EU), to economically disadvantaged areas in Northern Ireland and to regions close to each side of the border.

Science is one of the fund's beneficiaries and £2.5 million has been made available for research between 1995 and 1997. The money must be matched by local funds and will support three programmes: biotechnology; biomedical and environmental sensors; and rapid product development technology, which allows plastic models of any new product to be manufactured within hours.

The biotechnology programme is intended to raise the level of biotechnology in the north, where research is fragmented between small and relatively isolated groups, compared to that in the south. Through the activities of BioResearch Ireland, a government-backed contract research organization with five university-based centres, southern Ireland has developed a strong biotechnology infrastructure.

The IFI programme will create an analogous centre, known as the Centre for Innovation in Biotechnology (CIB), at two sites in Northern Ireland: Queen's University in Belfast and the University of Ulster at Coleraine. In addition, it will have an important strategic component, in that the programme will fund a detailed analysis of the strengths and weaknesses of biotechnology on both sides of the border, and an extension of BioResearch Ireland's database of biotechnology resources in Ireland to include the six counties of the north.

The new centre hopes to raise a further £1 million in industrial or EU funds during the three years it is supported by IFI, and then to survive chiefly on industrial contracts. Its first two scientific programmes, geared to exploiting local resources and industries, are intended to demonstrate the feasibility of this idea.

One, based at Queen's University, is designed to help mushroom farmers. Ireland has a successful mushroom industry on both sides of the border that uses waste

from the poultry industry, treated at central composters, to supply smallholders with growing medium for mushrooms.

In collaboration with the National Agricultural and Veterinary Biotechnology Centre in Dublin, the team at Queen's will study how to control outbreaks of disease which can lead to the destruction of crops and the need to quarantine land.

The research will involve monitoring the spread of outbreaks, studying the biochemistry of pathogenic organisms and developing diagnostic tests, based on DNA probes, to identify pathogens. "Pathogenic organisms don't recognize national borders", says Fred Wright, who heads the project at Queen's University. He argues that scientists should take the same attitude.

The second project exploits the spent fermentation waste from the brewing industry in the north to produce novel diagnostic products. Scientists at Coleraine will isolate and purify enzymes from the waste which they will then supply to BioResearch Ireland's laboratories in Galway.

The IFI biosensors programme has similar ambitions to the biotechnology programme. It combines the expertise of scientists from Queen's University and the



University of Ulster in the north, and others from Dublin City University and the University of Limerick in the south.

Dublin City University already has considerable expertise in developing biosensors, including a sensor that can be used to measure blood sugars (glucose and lactose) in real-time. Dermot Diamond, one of the researchers who developed the sensor, says that the IFI programme will help to overcome the previous lack of communication.

"We may not hear what our colleagues [in the north] are doing, even though Belfast is not many miles away," says Diamond.

Alison Abbott

Confident companies foresee biotechnology expansion in Europe

London. In contrast to the down-turn in the US biotechnology industry, Europe's biotechnology companies are continuing to expand and remain optimistic about their future prospects, according to an upbeat report that has just been published in London by the management consultants Ernst and Young.

Analysis of the response to a questionnaire from almost 200 companies using various types of biotechnology suggested that overall employment in the sector is expected to grow steadily, at an overall rate of about one per cent a year. Among smaller biotechnology companies, job prospects are even better, with an expected annual growth rate of six per cent.

Even in Germany, says the report, public opposition to biotechnology appears to be waning as awareness grows of the potential medical benefits of new biomedical technologies. This is reflected in moves by various regional authorities to support the growth of small biotechnology.

"European biotech companies are clearly more optimistic about the near-term future than are their US counterparts," says Bill Pike of Ernst and Young's National Biotechnology Office in Cambridge, England. Pike is one of the main authors of the second survey of the European biotechnology industry that was carried out in parallel to a similar survey conducted annually in the United States, and is based on responses to a questionnaire sent to more than 400 companies.

It points out that the 1995 US survey revealed that ten per cent of respondents indicated that one of their most significant actions over the next two years will be to lay off large numbers of employees. "No chief executive officer responding to our European survey anticipated such an action," the report states.

Challenging another widespread impression, the report points out that opposition to biotechnology in Germany is limited to a relatively few number of highly publicized protest activities. In contrast, in points out that a number of states — including Bavaria, Baden Württemberg and Rheinland Pfalz — have established biotech 'incubators' to help small companies get established.

The report describes this "new-found German enthusiasm for biotech" as "probably one of the most significant and encouraging changes taking place in Europe". It points out that Germany could become "a significant target for foreign companies hoping to take advantage of [its] excellent science base — and that "the idea that companies would choose to establish themselves in Germany was unheard of just a few years ago".

David Dickson

Pope condemns 'immoral' embryo research

Paris. In the strongest condemnation so far by the Roman Catholic Church of the ethical risks associated with modern medicine, Pope John Paul II last week condemned embryo research, medically assisted procreation and the use of prenatal diagnosis for achieving 'eugenic' ends as immoral.

The Pope's declaration comes at a time when bioethics legislation is being drafted by several countries. It seems likely to fuel debate about both the influence of the Church in this area, and the extent to which medical progress should be regulated either by society as a whole or by the conscience of individual physicians and researchers.

In his eleventh encyclical, *Evangelium Vitae* — 'The Gospel of Life' — the Pope reaffirms that human life begins at the moment of fertilization, and that absolute respect should be given to all forms of human life. The statement vigorously condemns abortion, euthanasia and artificial contraception, and prohibits *in vitro* fertilization because of the associated death of supernumerary embryos.

The authorization of euthanasia in the Netherlands is thought to have prompted the Pope to speak out, as he considers it the beginning of a slippery slope. Similarly, while the Pope "upholds as licit" manipulations of embryos aimed at healing rather than harming, he condemns research on embryos as a "crime against their dignity as human beings", and the use of embryos as a source of tissues and organs for transplants as "absolutely unacceptable".

The Vatican statement accepts prenatal diagnosis as a means for early therapy, or in order to "favour a serene and informed acceptance" of the child not yet born. But it condemns as a "shameful and utterly reprehensible" the eugenic goal of using prenatal diagnosis to allow abortion of fetuses suffering from abnormalities.

Such so-called 'genetic reductionism' — the tendency to stigmatize, ostracize or eliminate those not meeting a genetic norm —

also emerged as the biggest threat from genome research at a recent meeting of UNESCO's International Bioethics Committee (see *Nature* 371, 369; 1994).

While the Church's reaffirmation that life begins at fertilization will continue to provoke vigorous controversy, Jean-François Mattei, professor of paediatrics and medical genetics at the University of Marseille in France, says that this issue is "philosophical and theological".

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

Pope John Paul II: the encyclical reaffirms the Roman Catholic Church's opposition to embryo research.

"However you look at it, human embryos are fundamentally different from animal embryos", says Mattei, who claims that the real issue is about how society should incorporate moral issues into legislation.

France, which last year became the first country to introduce comprehensive bioethics legislation, shows how this issue can work out in practice. Mattei — who as a member of the French parliament strongly influenced the shape of the bioethics legislation — says that a basic principle is that a secular state cannot adopt a moral order where some impose their truth on others.

The Pope's stance represents only "Roman Catholic decisions about the Catholic faith aimed at Catholics," says

Mattei. He argues that morality and law have different roles to play. "But how can we choose between the possible, the desirable, and the forbidden without reference to moral, philosophical and religious values? If we have made much progress in acquiring knowledge, we have forgotten wisdom for too long."

Mattei and others also argue that decisions about the use of new medical techniques cannot be left to the conscience of researchers and physicians, as the "anarchy" of individual demands — for example, by patients — can "fundamentally modify the structure and evolution of society". Society, therefore, has a duty to provide legislation on such issues, says Mattei. He argues that "we cannot remain powerless in the face of aberrations that pervert progress".

The French bioethics legislation reaffirms the fundamental values of human dignity and the respect for other human beings from the beginning of life. At the same time, Mattei says the legislation is "realistic" in recognizing that exceptions need to be allowed.

The French parliament rejected attempts by the French senate, for example, to ban all research on human embryos, preimplantation diagnosis, and the destruction of supernumerary embryos produced in *in vitro* fertilization procedures.

This followed a vigorous debate in the French research and medical communities. Many participants, while divided on the real justification for much embryo research, argued that a "margin of liberty" should be given to the conscience of researchers and physicians. Similarly, the arguments of physicians that preimplantation diagnosis should be allowed for a defined list of very serious diseases was also accepted.

Declan Butler

Russian scientists struggle on against cuts in funding

Moscow. Yuri Osipov, the president of the Russian Academy of Sciences (RAS), said last week that for the first time in recent years there had been signs of stabilization in Russian science in 1994, reflected in the fall in the number of scientists of only 1.5 per cent compared to 4.5 per cent in 1992 and 3.6 per cent in 1993.

Osipov also said that although 1994 had been "a year of constant struggle and unfounded hopes", the academy had managed to maintain its integrity and "to remain the main centre of fundamental science in the state".

Addressing the academy's annual

meeting in Moscow, he said the academy's successes in 1994 had included the development of computers capable of running at more than 2 billion operations a second, and a new method of cancer therapy, using a combination of two harmless substances which, once targeted on a cancer cell, form free radicals that destroy it.

Osipov and other speakers also pointed out that the academy had been less affected by cuts in government spending than other sectors of the economy. In 1994 the academy had received 75 per cent of the amount originally planned through the Ministry of Science and Technical Policy,

while the allocations to other departments had been as low as 33 per cent.

Nevertheless, he described the way that the research money was used as "distorted", in that 48 per cent was spent directly on salaries, 35 per cent for electricity and government taxes, and only 17 per cent was spent directly on research.

Osipov confirmed that a lack of funding had meant that only one tenth of the scientific journals previously available can now be found in the research libraries, and that the academy had lost almost 3 billion rubles deposited in a bank that had subsequently collapsed.

Carl Levitin

Environment agency plans come under fire

London. The British government has rejected concerns that the proposed merger of three important public watchdogs into a single environment agency is a hasty measure and potentially harmful to science.

The merger, under the Environment Bill being piloted through Parliament, of the National Rivers Authority (NRA), Her Majesty's Inspectorate of Pollution (HMIP) and Britain's waste regulation authorities into a single Environment Agency from April 1996, is vital in developing a "holistic" approach to environmental problems, according to Environment Secretary John Gummer. The merger is designed to introduce cost-benefit analysis to environmental regulations. It will "engender a cultural change whereby environmental decision making will cease to be considered as an optional and costly extra duty", he says.

The government's critics, however, are far from satisfied with Mr Gummer's reassurances and are seeking rapid answers to

several key questions. The rhetoric of an 'integrated approach to solving environmental problems' has yet to be matched with workable proposals, according to David Coates, head of commercial affairs at the Natural Environment Research Council's Centre for Ecology and Hydrology. The government, he adds, must also clear up the present confusion over who will fund the new agency.

The government has promised £5 million in the agency's start-up costs. But it has so far refused to say who will fund the agency full-time. "A move to a multidisciplinary, holistic approach will need substantial investment in a programme for strategic research and development," says Coates. Yet up to now, "there has been no satisfactory answers as to where that investment will come from".

Industry is expected to foot some of the bill. But representatives, while agreeing investment is needed to enable the agency to

maintain credibility, do not see themselves fulfilling this role. Michael La Graff, general manager of health and safety at BP Chemicals, says industry should pay no more than "its fair share of environment protection costs and of the costs of the agencies".

Other fears yet to be allayed include concerns that the new agency will promote applied research at the expense of basic research. However Mervyn Bramley, head of research and development at NRA, says the individual agencies, when merged, will become a source of formidable research expertise.

"From the very first day the agency exists, it will draw on the scientific understanding that is the product of this cooperation [between the NRA and the HMIP and waste regulatory authorities], and the research the NRA has done over the past five years. The R&D work we are carrying out now will be crucial for sound environmental decision-making for the future." **Fiona Gammie**

Radiation study moves unsettle Japanese researchers

Tokyo. Officials of the US Department of Energy (DoE) visited Japan last week to try to calm the uproar generated by the department's plans to reorganize management of a joint US-Japan research foundation that has been studying the effects of radiation on atomic bomb victims for nearly 50 years.

This follows a letter sent by leading radiation biologists from the Universities of Tokyo, Kyoto and Hiroshima, as well as Nara Medical University, to Hazel O'Leary, US Energy Secretary, expressing concern about the DoE's plans.

The letter points out in particular that DoE is said to be planning to use the Radiation Effects Research Foundation (RERF), based in Hiroshima, as part of a training programme for the environmental cleanup of nuclear waste; it warns that this could hinder RERF's ability to continue its present long-term studies and damage the perceived neutrality and peaceful purpose of the programme.

In January, the department caused dismay among radiation biologists in both Japan and the United States by announcing plans to change the organization responsible for management of US participation in the foundation (see *Nature* 106, 374; 1995).

From its inception in 1947, the joint programme has been run on the US side by the National Academy of Sciences (NAS), and the academy's involvement has helped to win respect for the programme around the world. DoE now wants to transfer management of the programme to a US university or group of universities in order to attract young US scientists into research in radiation biology. But both Japanese and US sci-

entists associated with RERF fear this could jeopardize the programme in several ways.

Japanese scientists have been uncharacteristically outspoken in their criticism of the US move. In their letter to O'Leary, sent on 14 March, they echo comments of RERF



RERF: US plans 'could threaten its neutral position'.

scientists, saying that NAS acts as a "buffer" between DoE and RERF and provides "scientific credibility and cooperation of A-bomb victims essential to future RERF accomplishments".

Steven Galson, DoE's chief medical officer for environment, safety and health, who led last week's delegation to Japan, says there have been many "misconceptions" and admits that the announcement of the changes by DoE in January "could have been handled much better". But he insists the new arrangements will not impinge on the autonomy and neutrality of RERF, and that a university or group of universities can act as a "buffer" between DoE and RERF

in the same way as the academy does now.

Despite reports circulating in Washington, Galson claims that there will be no direct link between the training of young US scientists at RERF and the weapons clean-up programmes, although the results of RERF studies may well be applied in setting safety standards for the clean-up.

Another concern of RERF scientists is that DoE plans to ask the new administrative organization to evaluate the scientific objectives of the programme and make recommendations for its future in line with DoE objectives. This concern arises from an unsolicited proposal for management of the programme, sent to DoE by Columbia University in New York, which calls for such evaluation independently of RERF's own binational scientific council.

Galson says a Request for Proposals (RFP) for managing the programme will be announced in about a month. But it remains "unsettled" whether the RFP will include a component for evaluation or not. He says he will, however, convey to Washington the strong objections of RERF scientists to such evaluation. Under the RERF charter, according to DoE officials, a binational group of scientific advisers is responsible for the foundation's scientific programme, and this will not change.

Japan's Ministry of Health and Welfare, which administers the Japanese side of the programme, has said little on the issue. According to Galson, the ministry maintains a "neutral" stance and accepts that it is up to DoE to make arrangements for US management of the programme. **David Swinbanks**

UK, ESA and Integral

SIR — Giovanni Bignami (*Nature* 374, 110; 1995) accuses the United Kingdom, through the Particle Physics and Astronomy Research Council (PPARC), of “reneging on an agreement with the rest of Europe”, by failing to fund a substantial payload contribution to the Integral mission. In the same issue of *Nature*, Professor J. L. Culhane and others express the concern of UK scientists who expected to play a major role in Integral, adding that the anticipated costs of UK participation “were well known and [had] not changed substantially”. Perhaps I may be allowed to offer some background to this sad chapter in UK space science.

As the first chief executive of PPARC (and a space scientist by training), I am acutely aware of the damage to a section of the UK community, to the Integral project, and to Britain’s reputation as a reliable partner, resulting from our inability to play a substantial role in Integral. I also appreciate the impact on UK industry from the lost contracts in high-technology areas. However, the commitments and funding inherited by PPARC when it assumed responsibility for UK space science from the Science and Engineering Research Council a year ago led inevitably to the present outcome.

Funding of the ‘expected’ level of payload development by UK groups was estimated at £21 million. In contrast, the PPARC budget a year ago included only £3 million for that purpose, over the years 1995–98, with some expectation of an additional £7–£8 million based on the assumption that PPARC’s funding would be maintained, in real terms, over the following two years. Following its first full programme review, in September 1994, the PPARC council concluded that very limited funds were indeed likely to be available for the next two ESA missions, Integral and Rosetta. In an attempt to avoid reneging on agreements with colleagues elsewhere in Europe, I then made sure that the UK space science community and the European Space Agency (ESA) had early warning of our difficulties. Unfortunately, it does seem that these warnings went unheeded in the extensive proposals made to ESA in December.

As to the future, I strongly agree with Culhane *et al.* that it is vital that PPARC finds some way to ensure that the Integral debacle does not recur with subsequent ESA missions. Otherwise, UK membership of ESA would be called into question, as we became increasingly unable to enjoy the scientific and technological benefits of our subscription to this ‘world class’ space science programme.

To avoid such an outcome, which would be a disaster for UK space science, a considerable loss to an important sector of

UK industry, and damaging to the ESA programme itself, PPARC is actively working with its space partners in the UK and Europe to find additional funds for exploitation of future ESA spaceflight opportunities.

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Dissolubility of federations

SIR — While the bells of peace in Northern Ireland are beginning to peal and to be heard in Europe, your article on Russia’s heavy-handed approach to separatist tendencies in Chechnia and the principle of federalism could not have been more timely (*Nature* 373, 89–90; 1995). The continuation of the tradition of subjugation of people within sovereign states goes back a long way, particularly in Europe where countries are redefined after major wars. The concept of federalism means different things to different people and so is prone to misinterpretation. For those living in a federal state, it is an instrument of government that maintains cultural identity yet provides a central government to function economically and politically. The examples you have given of the Flemish and Walloon regions in federal Belgium are a paradigm of the two levels of government. But your points concerning Switzerland are not quite accurate.

Switzerland, although called a confederation, is since 1848 a federal state. A confederation is by definition an association of sovereign states ceding certain competencies to common bodies (for example, the Confederate States of America in 1861–65), whereas federal states such as Austria, Belgium, Germany, Switzerland or the United States of America today are composed of traditional/cultural territorial entities ceding to the federal government only functions such as foreign policy, currency and defence. Thanks to the federal system, the region of northern Jura, with a mainly Roman Catholic population, was able to separate from the canton of Bern and to become an independent new Swiss canton — whereas the southern Jura, mainly Protestant, remained with the canton of Bern. An example worthy to be given in the right context.

In conclusion, the question of how small states can survive can be answered by the example of Iceland. This proud nation of 300,000 people manages its place well in

Europe and has in the past taught lessons in independence to large states (the cod war!). The 25 years of military occupation of Northern Ireland by British troops only demonstrates that a central government cannot force cohesion between different communities within a country and therefore, maybe, a federal system might be better way for the future.

S. S. Baig

M. Hallen

Brussels, Belgium

War on anti-science

SIR — Like many other scientists, I agree with John Maddox (*Nature* 368, 185; 1994) that we have to be more active when struggling against the forces of anti-science.

In Russia there is now a rapid growth of publications and television and radio programmes dedicated to anti-science. Real scientific publications and broadcasting programmes seem of little account in comparison with the avalanche of anti-science. This is very dangerous for the education of the young in the evaluation of scientific and technical achievements and for the future of science and development of society as a whole.

As I see it, there are at least three reasons. (1) The press in Russia is no longer controlled by government censorship. As a result, all previously ‘hidden’ areas are blossoming in contrast to traditional ones. (2) Those working in astrology, alternative medicine and extra-terrestrial visitors are younger, more active, and more adaptable. They know the tastes of a wide public. (3) The language of anti-science is simple and more understandable for many people; anti-science speaks with confidence, whereas real science recognizes the limitations of knowledge.

So we need to find ways to popularize science and its new achievements. We need outstanding scientists who can explain complicated things using simple examples to attract the attention of young people to the beauties and secrets of nature.

It is necessary to demonstrate the difference between fact and fantasy, truth and lies, scientific forecasts and astrological horoscopes. We need to talk to the public in understandable terminology and with impressive examples. Television and radio companies would find time for useful scientific programmes if they were offered. It should not be a quick attack but a long siege, promising to provide good results many years ahead.

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Italian professorships

SIR — The most recent round of applications (*concorsi*) for full university professorship throughout Italy is just struggling to a close. It seems that things are at last changing, and that the move of protest is registering its first results.

Those who want the system changed say that the scandalous pressure on candidates, teachers and committees must be outlawed. Computerized analyses of scientific publications allow immediate comparison of applicants by objective parameters. The impact factor and citation index can no longer be ignored as objective approaches to evaluation of scientific candidates. Two examples (see figure) demonstrate the clear superiority of our record compared to the majority of the successful candidates. Our applications were nevertheless unsuccessful. To add insult to injury, the weakest winners were

collaborators of commission members — the usual outcome of the present *concorsi* system.

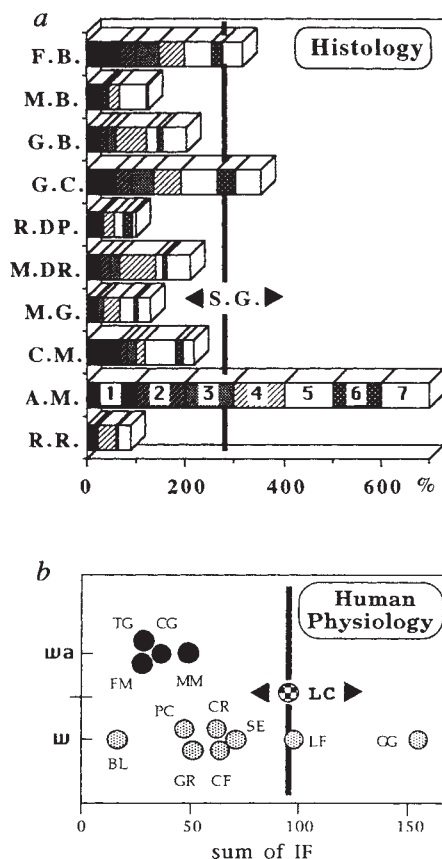
We, together with others from other faculties, organized a series of national meetings, which led to the creation, in mid-February, of the Academic Research and Culture Association (ARCA). According to press reports, the public prosecutor of Rome has opened an enquiry into a number of *concorsi*, on the basis of our dossier. The universities of Rome, Padua, Cagliari, Milan, Florence and Naples, which are under investigation, have cause to be concerned, given the criminal conviction of the medical faculty commission chairman for abuse of authority.

Prevention of abuse is equally if not more crucial. It is here that ARCA will have a role, by promoting the best academic candidates in Italy. ARCA will recommend approaches that include the use of objective parameters in the selection procedures for academic promotions and distribution of research funds, in the assessment of academic output at all levels, and in the setting up of tight controls over commission membership. Padua university has opened the way: medical faculty rules approved on 23 February set an 'impact factor' threshold for future members of its scientific and development commissions, and its promotion commission is now adopting objective parameters.

The universities in Italy, with their newly acquired financial and managerial autonomy from the research ministry, will have to improve their cost-quality ratios to survive in the national and international arena, and this in turn should restore the cultural and ethical basis on which they were originally founded.

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a. The scientific weight of the ten histology winners, expressed as percentages of leader's values (A.M.), and compared to the weight of the present author S.G.: (1) number of papers, (2) sum of the impact factors (IF*), (3) sum of normalized IFs (IF/number of co-authors), (4) average IF, (5) number of papers as first or senior author (FS), (6) sum of IFs as FS, (7) average IF as FS. b. The scientific weight (as total IF) of the 12 human physiology winners (black and grey circles; w + wa) compared to the writer L.C. The winners indicated by the black circles (wa) were collaborators of the committees.

Such statements as "seismic waves themselves do not kill people in large numbers. But buildings and other components of the built environment do", or "Experience . . . has amply shown what benefits can accrue from buildings designed against earthquakes" conceal a fundamental logical inconsistency. Engineers cannot design earthquake-resistant buildings without knowing how the ground is likely to shake when an earthquake occurs. Building codes are based on estimates of ground motion. Ground motion is determined by the shallow geological structure beneath buildings, the deeper structure between the earthquake and buildings, the location of the earthquake relative to the building, and properties of the earthquake source. Knowledge of these factors comes from seismological studies.

Engineers and scientists alike will agree that where this information is accurate and appropriately accounted for in the building codes, lives have been saved. Indeed, the destruction following the Kobe earthquake would have been far worse had there not been this partnership between seismologists and engineers.

As you correctly suggest, the costs of building seismically safe structures (where 'safety' is defined relative to the likely seismic motions, assuming such estimates exist) may be extremely high. Seismic zonation, which quantifies the variability in probable earthquake occurrence and consequent shaking, provides a demonstrably effective way to minimize this cost. This is the realm of seismology, not engineering.

It is short-sighted to imply that seismology has failed because experimentation in earthquake prediction has not yet resulted in "the prize seismology should deliver". Outside Japan, research for explicit earthquake prediction experiments receives a relatively small part of monies spent on seismology. Societies spend orders of magnitude more on the search for a general cancer cure though most would agree that it, too, is "an impossible dream for the present".

Like the failure to find a cancer cure, the failure to predict an earthquake does not signify a failure of earthquake prediction research. Rather, the research process has yielded valuable advances in our understanding of the causes and effects of earthquakes, understanding useful for both short- and long-term mitigation of earthquake hazard.

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Earthquake hazard

SIR — Your rather glum conclusions about the seismological lessons from the Kobe earthquake (*Nature* 373, 269; 1995) are off base to the extent that they imply that seismology has little to offer hazard mitigation, and that more engineering is the answer.

Lophophorates prove likewise variable

Evidence from molecular phylogeny challenges the conventional wisdom that places an obscure but pivotal group of organisms close to our own ancestry.

BRACHIOPODS were once called lamp shells, because they look like the lamps seen in productions of *Aladdin*. Like Aladdin's magic lamp, brachiopods have a genie inside. It is a feeding structure called a lophophore, a horseshoe-shaped arrangement of ciliated tentacles surrounding the mouth. This organ marks brachiopods as different from bivalve molluscs, which they resemble only superficially. But lophophores are also found in bryozoans, and marine worms called phoronids. Tradition has grouped these three phyla together on the basis of the shared presence of the lophophore, on the grounds that such a complex organ is unlikely to have evolved more than once.

Fitting the lophophorates into the larger scheme of things is more difficult. Animals more complex than flatworms conventionally belong to one of two large groups, the protostomes or the deuterostomes. Like Montagues and Capulets, the differences between the two are ontogenetic and hard to tell from adult appearance. In protostomes, the hole in the end of the blastula becomes the mouth, cleavage between the cells of the early embryo tends to be spiral, development is determinate, and the coelom (or body cavity) originates by the splitting of pre-existing mesodermal layers. Prominent protostomes include molluscs, arthropods and annelids: *Drosophila* is a protostome.

In deuterostomes, in contrast, the hole in the end of the blastula becomes the anus and the mouth develops separately (whence 'deuterostome', or 'second mouth'), cleavage is radial, development indeterminate, and the coelom develops at the same time as the mesoderm, from infolding regions of the primitive gut. The coelom in many deuterostomes is tripartite, arranged as three cavities from front to back. The second and third coeloms, if not always the first, are in turn subdivided into left and right halves. Deuterostomes include echinoderms and chordates: the best known is *Homo sapiens*.

Cleavage in lophophorates is generally thought to be radial, and the coelom is usually held to evolve from infoldings from the gut. The coelom also tends to be tripartite (although the presence of the foremost compartment is debatable), and the two halves of the lophophore originate from the two halves of the second pair of coelomic cavities. It is along these lines that lophophorates have been allied with the deuterostomes.

Embryological differences, though, are not always as clear-cut as these simple definitions imply. To complicate matters, lophophorates exhibit features that suggest a protostome affinity. Brachiopods, for example, have chitinous, hair-like structures similar to the setae of annelids.

If conventional wisdom has it that deuterostomes represent a monophyletic pearl amid a paraphyletic, protostome oyster, then the lophophorates can be placed as the nacre closest to the pearl, and yet not of it. Although protostomes, they occupy a special place as those closest to the ancestry of deuterostomes, having acquired features associated with deuterostome ontogeny, but not yet having lost primitive traits of protostomes.

And so it would stand, were it not for the unquiet stirrings of molecular evidence. Phylogenetic reconstruction based on ribosomal RNA gene sequences has allied brachiopods with molluscs. But the evidence gathered hitherto has been tentative and easily dismissed as more protostome primitiveness.

Perhaps no longer. Complete 18S ribosomal RNA genes are now available for many species. Kenneth M. Halanych and colleagues use them to show that lophophorates are grouped firmly within the protostomes (*Science* **267**, 1641–1643; 1995). Several different methods of reconstruction reach essentially the same result. Lophophorates are not just protostomes but advanced protostomes, and have no special relationship with deuterostomes.

In the consensus tree presented by Halanych *et al.*, protostomes emerge as a strongly supported clade, but the deuterostomes (in this analysis a crinoid, an amphioxus and a lamprey) group together far less well. Within the protostome group, the arthropods branch off first and (now, this is the interesting part) the bryozoans come off next.

This leaves a 'crown' group of brachiopods, phoronids, molluscs and annelids. Within this crown, no species is closer to any other, except that phoronids emerge as closer to articulate brachiopods (in which the shells are hinged together) than inarticulate ones (in which they are not).

In general, the result has fascinating implications. First, the presence of the lophophore defines a subgroup of the protostomes (which the authors call the Lophotrochozoa) that includes the lophophorates, molluscs and annelids,

but excludes arthropods.

Second, the position of bryozoans, which are lophophorates and yet primitive with respect to annelids, molluscs and the other lophophorates, suggests that annelids and molluscs had a lophophorate ancestry, but their affinities have been masked by the secondary loss of this definitive structure.

Critics can hardly balk at the suggestion that annelids and molluscs are lophophorates that happen to have lost their lophophores, for the traditional alignment of lophophorates with deuterostomes requires that chordates and echinoderms should have suffered the same loss.

Nevertheless, one is entitled to wonder whether Halanych *et al.* are justified in throwing out much ontogenetic and morphological evidence on the basis of the sequence of a single molecule: particularly as there is another group of lophophore-bearing animals, the pterobranchs, which do not feature in their analysis.

Like the lophophorates, pterobranchs are small, obscure sea creatures. Only three genera are known, but all have lophophores derived from the second pair of coelomic cavities. Yet zoologists do not group pterobranchs with lophophorates, but with the acorn-worms or enteropneusts, which lack lophophores. Pterobranchs and enteropneusts together comprise the hemichordates, and are deuterostomes. If the lophophorates are protostomes, as Halanych *et al.* suggest, then the lophophore must have evolved twice independently. The extent to which molecular evidence from hemichordates would dent the monophyly of the new-made Lophotrochozoa remains to be seen. And there is one further piece of evidence to be considered. It comes (one will no longer be surprised to learn) from yet another group of obscure sea creatures, this time one with which even zoologists are unlikely to have made much acquaintance. From the abyssal depths of the bathyal ooze has emerged a variety of acorn-worm of unusually piebald character. Although they look like the acorn-worms from inshore waters, they sport lophophore-like fringes of tentacles growing from a region that corresponds with the second pair of coelomic compartments. These animals are almost certainly deuterostomes, yet a lophophore by any other name would smell as sweet. Whether the same applies to the Lophotrochozoa is less certain.

Henry Gee

Protease uninhibited

Douglas D. Richman

THE intense media coverage of AIDS puts the public on an emotional roller coaster: inflated promises are engendered by each success and predictions of hopeless failure with each setback. The paper by Condra *et al.* on page 569 of this issue¹, in which the authors describe the emergence of HIV-1 variants that are resistant to several protease-inhibitor drugs after treatment with just one, is likely to be interpreted as a big 'down'. The findings do indeed come as a setback, but the implications are not as gloomy as they might seem.

The drug used by Condra *et al.* was MK-639, which inhibits the protease activity of HIV. Just three months ago, two studies with MK-639 and ABT-538, a drug with similar antiviral characteristics, generated important observations about the population dynamics of both HIV and CD4 lymphocytes in the circulation, and about the efficacy of antiviral therapy^{2,3}. Progress and encouragement came from the realization that high rates of destruction of CD4 lymphocytes are matched by similarly high rates of production of uninfected cells; that the high rates of production of HIV are accompanied by rapid rates of virus clearance; and that potent inhibitors of viral protease activity can rapidly reduce virus replication by as much as 1,000 times and restore CD4 cell counts, even in patients with advanced disease.

Resistant virus

Now for the setback. Condra *et al.* report that within 6–12 months this anti-retroviral activity is largely dissipated in conjunction with the acquisition of highly resistant virus. Although disappointing, many aspects of these observations are not unexpected. On the one hand, highly resistant mutants of HIV appear to emerge with difficulty following exposure to zalcitabine (ddC), didanosine (ddI) or stavudine (d4T), dideoxynucleosides with sugars that resemble their physiological counterparts^{4–6}. On the other, high-level resistance readily develops with other nucleosides (zidovudine (AZT) and lamivudine (3TC)), non-nucleoside reverse-transcriptase inhibitors and protease inhibitors⁷.

The mutability of the HIV protease is remarkable. Some 20 of the 99 amino acids in each polypeptide of this homodimeric enzyme have been shown to undergo mutation when faced with the selective pressure of various inhibitors^{8,9}. Condra *et al.* now find that the cumulative acquisition of six or more of these mutations is possible, resulting both in 1,000-fold reductions in viral drug-susceptibility and in broad cross-resistance to other

inhibitors against which cross-resistance is not observed with fewer mutations. As the authors point out, X-ray crystallographic models of the enzyme locate many of the mutations in the protease pocket which binds the inhibitor, thus reducing its binding affinity^{10–12}. Other mutations at distant residues must result in conformational changes that either contribute to impaired binding or compensate for deleterious effects of drug-resistance mutations on enzyme function.

The cumulative acquisition of mutations over time within a patient, and the different evolutionary pathways that the virus takes in different patients, should not be surprising. Progressively greater resistance requires the accumulation of additive or synergistic mutations. In accommodating these mutations, the enzyme cannot tolerate compromises in its function. Such compromises would attenuate the replicative capacity of the virus. Coffin¹³ has argued clearly and cogently that small impairments in viral fitness would result in evolutionary failure during an infection that is characterized by high levels of virus with rapid rates of turnover. Thus many mutations compensate for impaired virus replication rather than contribute to the drug-resistance phenotype¹¹. The different combinations of mutations that emerge may result partly from chance and partly from the baseline sequence of the virus before drug treatment. Several investigators have presented data, not yet published, that the amino-acid sequence of different isolates before treatment affects the phenotypic effects of different drug-resistance mutations.

What is the clinical significance of these observations? Unfortunately Condra *et al.* do not provide any information regarding the clinical status of their patients, or the viral responses (and loss thereof) with treatment. Nevertheless, the message the authors seem to convey is that monotherapy with MK-639 has no future: it abrogates the potential benefit of treatment with alternative protease inhibitors, and "multiple protease inhibitors may not prevent loss of antiviral activity resulting from resistance selection". This seemingly hopeless message, based upon four patients, is open to question. Not least, it has not stopped Merck, for whom most of the group work, from continuing to develop MK-639. It should not stop others.

There are at least three theoretical mechanisms by which antiretroviral drugs can sustain activity in the face of drug-resistant virus. First, drugs can continue to exert antiretroviral activity if plasma concentrations can be maintained

that exceed the susceptibility of drug-resistant virus (assuming, of course, that there are constraints on the mutability of the target protein). This approach appears to apply to some patients in whom high levels of the non-nucleoside reverse-transcriptase inhibitor, nevirapine, can be achieved¹⁴. Second, drug-resistance mutations, which confer a clear selective advantage in the face of drug pressure, may still impair the replicative capacity of the virus compared to that of the wild-type virus in the absence of treatment. Such attenuated virus may contribute to the activity of lamivudine and perhaps of multiply resistant protease mutants.

Convergent therapy

Third, when two drugs are targeted to the same viral protein (convergent therapy), mutations induced by drug 1 may sensitize the virus to drug 2, or may prevent the emergence of viable mutants to drug 2. The mutation from methionine to valine at residue 184 of reverse transcriptase, which emerges with lamivudine treatment, suppresses the critical mutation at residue 215 that confers resistance to AZT (ref. 15). Combinations of protease inhibitors may also exploit this strategy. It seems logical that a potent inhibitor can be designed to bind to the active site of the multiply resistant viruses described by Condra *et al.*

Recent insights about the targets for antiviral drugs and the pathogenesis of HIV infection provide a more rational basis for developing effective treatments. Given that some people who have had long-term HIV infection have not developed AIDS, we know that a significant but incomplete suppression of virus replication is a reasonable initial aim which, if achieved, should allow patients a greater time free of disease. Setbacks are to be expected during the trek towards this goal — but they should not discourage the endeavour. □

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Hollow organic solids

Jeffrey S. Moore

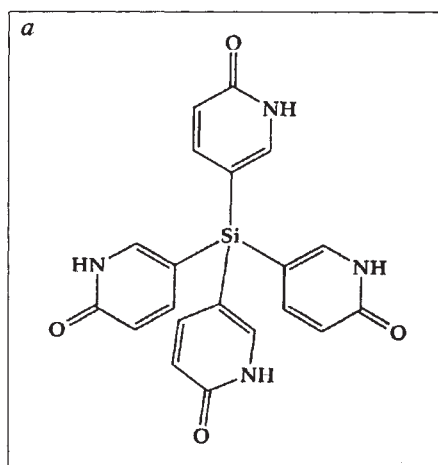
To perform chemical reactions or separations with high specificity, the random environment of a solution is not ideal; it can be better by far to trap one species in the regular hollows of an organized medium. Crystalline solids are the pinnacle of molecular order and may provide chemical reactors of extraordinary selectivity. But with most crystalline materials, closest packing provides an overriding driving force that precludes the formation of open, molecular-sized hollows — the working space for chemistry or separations. When open features do occur in molecular solids, they are typically filled with tenaciously bound guest molecules. In rare instances where solid-state hosts trap labile guests, the crystal is usually extremely fragile, so much so that loss of the guest immediately leads to destruction of long-range three dimensional order. Now, though, J. Wuest and co-workers¹ have described the design and preparation of a crystalline organic host that not only forms large molecular channels but also accommodates labile guests that can be reversibly exchanged without loss of crystallinity.

Closely related to the materials described by Wuest and his colleagues are inorganic zeolites — open-pore aluminosilicates and aluminophosphates that form three-dimensional covalent networks. The advantage of constructing organic nanoporous solids is that the shape and functionality of the building block can be widely varied, unlike the inorganic constituents used to make traditional zeolites. With an organic-based approach to zeolite-like structures, there is strong reason to believe that solids could be rationally designed, especially as chemists' command of supramolecular interactions improves. Because of the much weaker nature of the noncovalent forces that hold molecular solids together, however, there is also cause for scepticism as to whether organic-based zeolites could ever function quite like their inorganic counterparts. The report by Wuest and colleagues goes some way towards laying these uncertainties to rest.

Using the structural analogy of inorganic zeolites, Wuest and others before him^{2,3} have shown that the way around the closest packing problem is to base solid-state design on extended network structures. Supramolecular networks with strong, selective and directional noncovalent interactions such as hydrogen bonds or metal coordination bonds can provide enough of a bias to steer molecular organization away from closest packing. To make the network extend in three dimensions, the junction points must be at least

triply connected. Wuest calls the molecular building blocks that form these junctions 'tectons'. Using a tecton with hydrogen-bond-forming groups placed on the ends of a tetrahedral frame (part *a* of the figure) results in the formation of an extended diamondoid network. The structure has large chambers whose size and shape follow directly from that of the tecton.

But nature is clever in finding ways to fill this empty space. One tactic involves regular interpenetration of networks through mutual concatenation during growth of the crystal (part *b* of the figure).



a, Wuest's molecular tecton contains tetrahedrally oriented pyridone rings and crystallizes into open, three-dimensional hydrogen-bonded networks. *b*, To fill the large void volume that would be left by one network, a second, identical diamondoid network interpenetrates it (to distinguish them, the two networks are coloured as green cylinders and red space-filling balls). This section of the network shows that the intertwined nets leave behind square channels that extend throughout the lattice. The space within these channels is occupied by labile but fairly well ordered solvent molecules ($\text{CH}_3\text{CH}_2\text{COOH}$).



The network's large open space vanishes as it becomes filled with offset, but otherwise identical, copies of the original network. It has been widely believed by researchers in the field that interpenetration precludes the formation of channel structures. To circumvent this problem, chemists have devised approaches using ring-like building blocks that cannot form self-filled networks⁴.

In the case of the crystalline solids reported by Wuest's group, a pair of interpenetrated diamondoid networks resulted from the tetrahedral tectons. This degree of interpenetration does not, however, fill all available space, but leaves large square channels ($6 \times 6 \text{ \AA}$) that extend throughout the lattice. It seems that there is not enough room to accommodate a third network. These findings show that interpenetrated networks do not always preclude the formation of extended channel structures. The channel volume in the doubly interpen-

strong on the scale of non-covalent interactions, they are not much different from those of other hydrogen-bonded crystalline hosts. It is interesting to speculate that part of the stability arises from the intertwined nature of the two independent concatenated networks. But in comparison with zeolites, the stability of Wuest's molecular-derived zeolites is low. As the authors are careful to point out, their crystals are destroyed if the guest species are removed without being replaced by another molecule in the empty space.

For the goal of molecularly designed, shape-selective solid catalysts there is still a way to go. Although the topology of a single network can reasonably be predicted from the geometry of its constituents, it is nearly impossible to predict the fine details of the interpenetrated structure. This is further complicated by polymorphism — the nemesis of crystal engineering — where the subtleties of kinetically controlled nucleation dictate

which of several energetically similar crystal structures will form. Moreover, Wuest's materials do not have any built-in component that could perform catalytic chemistry. Others have made progress towards this objective. For example, Richard Robson of the University of Melbourne has constructed porous metal coordination networks based on a porphyrin building block known to have catalytic activity⁵.

Even though there is a long way to go before organic zeolite analogues come to fruition, Wuest's discovery demonstrates an essential step. The essence of chemical reactivity is the ability to bring together a substrate and a reagent through molecular diffusion. We now know that this diffusion can occur on a reasonable timescale through a nanoporous organic crystal.

Although organic zeolites will never be as robust as their inorganic counterparts, they can still be useful so long as molecules can enter and leave the shape-selective pores without causing the lattice to crumble. □

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MOLECULAR BIOPHYSICS

Taking a shine to myosin

Robert M. Simmons

SOME 20 years ago Hirschfeld¹ detected the fluorescence from an antibody labelled with about 80 fluorophores. As reported by Funatsu *et al.* on page 555 of this issue², techniques have now been improved to the point where it is possible to detect single fluorophores using a video-camera with a light microscope, with good resolution of intensity and time. Similar achievements in focused beam experiments have already been reported^{3,4}.

Detecting single-molecule fluorescence in solution has presented a challenge because of scattering from the solution. But the rewards for success are considerable in three main areas.

First, there could be some interesting physics of the excitation–emission process itself, which might be averaged out in an ensemble of fluorophores. Second, and this is the major impetus, it might be possible to sequence single molecules of DNA: the notional experiment is to make complementary DNA with bases having different fluorophores, tether the molecule in a capillary tube and digest it with an exonuclease while flowing solution along the tube; then, with a sensitive detector downstream, to distinguish between the fluorophores by spectral or lifetime differences. The research on spectroscopy and sequencing has concentrated on reducing the volume illuminated by the laser beam used for excitation, so as to minimize the background scatter, and on improving the efficiency of detection. The detection of single-molecule fluorescence was inferred from correlation analysis ten years ago⁵, but it is only recently that bursts of photons have been shown convincingly to arise from single fluorophores traversing the laser beam. It has also been

possible to measure lifetimes, using a pulsed laser and measuring the delay before a photon is emitted⁴.

The third reason comes from motility assays in which the action of motor proteins is studied *in vitro*. One example is the imaging of actin filaments sliding over a glass surface coated with the motor protein myosin. Experiments in which single motor-protein interactions are detected^{6,7} would be greatly enhanced by positional information about the myosin molecules and the biochemical kinetics of the interactions. One goal is to identify unambiguously the position of single motor-proteins, from which mechanical measurements can then be made. This would resolve lingering uncertainties about how many molecules are needed for an effective interaction. A video-microscopy technique is needed for the purpose, one that can tell the difference between one fluorophore and two, and which has a video-rate temporal resolution.

Funatsu *et al.*² have now achieved this end using conventional instrumentation, but taking extreme care to reduce background scattering and fluorescence. Measured over the area of resolution of a single fluorophore, these precautions reduced the background scattering from more than 6,000 photons per second to 140 photons per second, to be compared with 500 photons per second from the fluorophore itself. Background counts were then finally nearly abolished using an evanescent-wave technique to illuminate the surface on which the fluorophores (attached to myosin molecules) were located. Lastly, electron micrographs of the myosin molecules on a surface superposed

well on the fluorescence image.

Game and set, if not match, in single motor-protein studies would be won by simultaneous measurements of mechanics and biochemical kinetics. This could settle once and for all the issue of which biochemical transition accompanies force generation (popularly thought for myosin to be phosphate release). It would also lay to rest (or resurrect) the spectre of multiple interactions of myosin with actin for each ATP hydrolysed⁸. The first experiments towards this end were reported two years ago by Sowerby and colleagues⁹, who measured single turn-overs of ATP in myosin filaments containing about 10⁴ molecules.

Funatsu *et al.* have brought these measurements to the single-molecule level, using a fluorescent analogue of ATP at low concentration (10 nM), and watching molecules of the analogue bind to myosin molecules and then disappear again as the ATP analogue is hydrolysed and the products diffuse away. One limitation is the need to use very low concentrations to avoid high background counts, although, as Sowerby *et al.*⁹ point out, it may be possible to use a photoactivatable fluorophore and photolyse it locally. Another limitation is the comparatively poor time resolution, arising from the low photon detection rate; in all these single-fluorophore measurements, it is tantalizing that the detection rate appears to be 1 per cent at best³, and also that the number of photons is limited to about 10⁵–10⁶ by photobleaching¹. So it seems that as few as 10³–10⁴ photons are available for measurement, necessitating a compromise between temporal resolution and duration.

Game, set and match in single motor-protein experiments would be to measure the mechanics, biochemistry and structural changes accompanying force generation in the same experiment. If this were to involve both fluorescence and a scanning-probe technique, one might be tempted to say that there was — at last — light at the end of the tunnel. □

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Many wheels make light work

Werner Kühlbrandt

PLANTS and some bacteria have the unique ability to fix solar energy by photosynthesis — but how? The mechanisms underlying this most fundamental of life processes are being unravelled one by one as we gain insights into the molecular architecture of the photosynthetic machinery. The atomic structure of the bacterial light-harvesting complex LH2, described by McDermott *et al.* on page 517 of this issue¹, is an important step along the way. It makes a satisfying complement to the structure of the bacterial reaction centre, worked out by Michel and colleagues² in 1985. In the reaction centre, a radical ion pair is generated which withdraws electrons from a substrate and transfers them to an acceptor on the other side of the membrane. The energy for this reaction is collected by pigment molecules attached to the light-harvesting complexes. Knowing what both assemblies look like at the atomic level, we are now close to a complete picture of bacterial photosynthesis.

The structure described by McDermott *et al.* is both simple and elegant: nine identical units, each consisting of two fairly short α -helical polypeptides and associated pigment molecules, combine into a ring. The helices are perpendicular to the plane of the membrane, or slightly tilted, as was found in three other α -helical membrane proteins²⁻⁴. Within one unit, each pair of polypeptides coordinates three bacteriochlorophylls and one carotenoid. Two bacteriochlorophylls are held near the periplasmic, carboxy-terminal end of the helices, and one is suspended between the helices near their middle. Within the complex, there are thus two rings of bacteriochlorophylls, one set of 18 close to the membrane surface, arranged as in the wheel of a turbine, and another set of nine in the middle of the bilayer. Their chemical environments are different, so they absorb at different wavelengths, extending the spectral range of light harvested.

Most photosynthetic bacteria have two or more light-harvesting complexes with distinct biochemical and spectroscopic properties. LH2 is sometimes referred to as the outer antenna because the energy it collects flows to its final destination through another, inner antenna complex (LH1) which immediately surrounds the reaction centre to which the light energy must ultimately be transferred (see figure). The structure of this inner antenna complex is being investigated by electron crystallography of two-dimensional crystals, rather than X-ray crystallography of three-dimensional crystals, the technique used by McDermott *et al.* It so happens

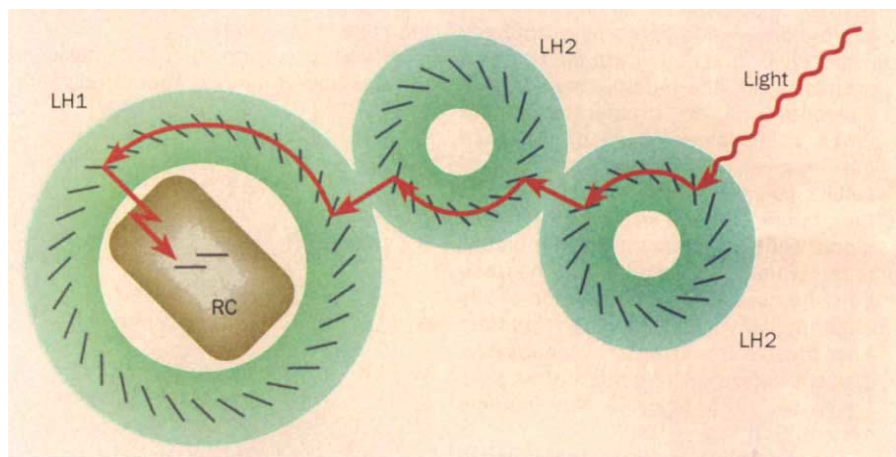
that a projection map at 8.5-Å resolution of LH1 has just been published⁵ which shows that this complex also has a ring-like structure, and is built up from the same type of molecular units as LH2. The LH1 ring contains 16 rather than nine units and is therefore considerably wider, to accommodate the reaction centre.

It is intriguing that the number of subunits in the ring of both complexes is variable, as indicated by another form of two-dimensional crystals of LH1 which consists of smaller rings⁶, and by the observation that some crystal forms of LH2 are incompatible with nine-fold

single photon is conducted in a very short time, and with minimal loss, from the point where it is absorbed to where it is needed.

Because of the circular shape, the flow of excitation energy from one antenna complex to another does not depend on their rotational orientation, as McDermott and colleagues point out. The arrangement of the antenna chlorophylls also explains why energy transfer to the reaction centre is so fast and efficient: the 'special pair' of bacteriochlorophylls in this complex is located at the same level in the membrane as the antenna pigments, and is thus perfectly poised to receive the energy collected in the rings around it.

Surprisingly, the bacterial light-harvesting complexes bear no structural resemblance to the plant light-harvesting



The structure of the bacterial light-harvesting complex LH2 suggests how absorbed light energy passes through the similar LH1 complex to its final destination, the special pair of bacteriochlorophylls in the reaction centre (RC), where it is used to propel an electron across the membrane. The diagram shows a view from above the membrane.

symmetry⁶. Apparently, the molecular design of these complexes is modular, because rings of various diameters can form from the same subunits. A comparison of polypeptide sequences shows that the binding site for the chlorophyll in the centre of the membrane is missing in LH1, whereas the pairs of histidine residues which ligand the other two are completely conserved. So arrangement of the bacteriochlorophylls in the periplasmic ring is likely to be the same in both forms.

When a photon hits one of the chlorophylls, the absorbed energy spreads extremely rapidly (in less than one picosecond) to the others in the ring, on account of their favourable spacing and orientation. Where the rings touch in the close-packed membrane, the energy can easily jump the short distance to an adjacent complex where it again spreads in the ring. LH1 absorbs at a longer wavelength and, hence, lower energy than LH2. It serves as an energy funnel for the reaction centre which, having the most redshifted absorption maximum, acts as an energy sink. In this way, the energy contained in a

complex⁴ (LHC-II), even though some governing principles of construction, in particular the distance between chlorophylls and their close association with the photoprotective carotenoids, are similar in both cases. LHC-II is much larger, binding 12 or more chlorophylls and two carotenoids per polypeptide, and lacks the repetitive simplicity of LH1 and LH2. The two complexes represent quite different answers to the same biological problem of efficient light absorption and energy transfer in a membrane.

Although the first crystals of the bacterial antenna complex solved by McDermott *et al.* were grown nearly ten years ago, their structure has proved difficult to crack. As with other membrane

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proteins, it took a great deal of careful work to improve the quality of the crystals, and some ingenuity to determine their structure. Subtle changes in unit-cell dimensions occurred when the crystals were soaked to obtain heavy-metal derivatives necessary for phase determination. The authors finally resorted to a trick referred to as 'back soaking' to dislodge

one of the heavy metals from its binding site. The problems associated with the unit-cell change could thus be avoided, and at last the structure was solved.

As with reports of every structure of a membrane protein — the total is now up to six — news that the structure of the bacterial light-harvesting complex has been solved will be greeted with the

particular sense of excitement we feel when the curtain is finally raised on a long-awaited scene. The stage is now set for further revelations about the way in which living organisms make light work. □

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OBITUARY

Georges Köhler (1946–95)

ON 1 March Georges Köhler died of heart failure while suffering from pneumonia. He was only 48.

Together with César Milstein, Köhler invented the technique for producing monoclonal antibodies. This method uses the capacity of an antigen-activated normal B lymphocyte to produce and secrete one — and only one — antibody with a given specificity, and the ability of a tumour cell, the multiple myeloma or plasmacytoma, to proliferate without limits at the same stage of B-lineage development. Following fusion, the resulting so-called hybridoma grows like the tumour cell and secretes the monoclonal antibody in any desired quantity. In principle, this method allows the isolation and expansion in production of any antibody within the seemingly limitless and diverse repertoire of antibodies, present before or generated after challenge by an antigen in the immune system.

The generation of hybridomas is one of the most powerful inventions of the past 20 years. Monoclonal antibodies are reagents for the identification and purification of molecules and cells, both in basic research and in medicine. They have raised fresh hopes for Paul Ehrlich's idea that targeted antibodies can be used as magic bullets against infectious diseases and cancer. For inventing them, in 1984 Köhler and Milstein received the Nobel Prize in Physiology or Medicine, together with Niels Kaj Jerne who shared the prize for his theories concerning the specificity in development and control of the immune system.

As a PhD student of the University of Freiburg, Köhler studied the diversity of the immune response against the enzyme β -galactosidase in my laboratory at the Basel Institute for Immunology. In 1974, when he finished his thesis, it was already known that single cells of the immune system produce only one type of antibody. But the genetic basis for this antibody diversity had not yet been discovered. Jerne, then director at the Basel Institute, had proposed that diversity might be generated by somatic mutations of a limited number of antibody genes transmitted from generation to generation. Köhler's plan was to study

these mutations in antibody-producing cells by observing the clonal growth from one cell to thousands and millions of cells, all producing the same 'monoclonal' antibody. Unfortunately, however, normal antibody-producing cells grow only for a few divisions before they die — too few to detect any changes in one of the cells in the clone.

Milstein's laboratory in Cambridge, UK, was one of the few where malignant



Georges Köhler — co-winner of a Nobel prize.

forms of these antibody-producing cells were grown in tissue culture, almost like bacteria, for unlimited periods of time. However, the antigen-binding specificity of the antibodies produced by the tumour cells was unknown. In 1974 Köhler received an EMBO fellowship to join Milstein's laboratory to look for mutations in antibody-producing cells. In long and lively discussions with Milstein, Köhler developed what he himself called the "crazy" idea of fusing a normal antibody-producing cell with a tumour cell at the same stage of development. He decided to use sheep erythrocytes as the antigen, because cells secreting antibody specific for this antigen could easily be detected as single cells in Jerne's haemolytic plaque assay.

He was lucky twice. First, he was fortunate to choose normal antibody-producing cells from a mouse that he had activated by immunization before his attempts at fusion. It turned out that

the activated cells fuse 100 times better than non-activated cells, meaning that the chances of finding the desired hybridoma were likewise increased. The second stroke of luck was that the hybrid cells were stable and continued to produce antibody, in contrast to the results in previous fusion experiments by others. Never before had fusion partners kept the capacity to produce antibodies. Köhler's insight and good fortune gave him the cells to study how antibodies are made — a complex and exciting project which he pursued until his death.

In 1976, Köhler returned to the Basel Institute, where he continued his studies on the basic mechanisms of antibody generation and production in the cells of the immune system. Monoclonal antibodies have developed into a multibillion-dollar market, but neither Köhler nor Milstein participated in it. They did not patent the technique, nor did they found a company. Both of them remained dedicated to basic research. In 1985 Köhler left Basel to become director at the Max Planck Institute for Immunobiology in Freiburg. There he entered a second research area, the control of immune responses by cytokines.

Georges Köhler was devoted to science. He used his influence as a Nobel prizewinner to plead for the support of basic research motivated neither by commercial nor by other practical interests. He defended the freedom of science in society, and the principle of the need to know. When asked whether he didn't think it better to stop some research that could yield results which might have dangerous applications, he responded "Yes, we must control the applications of our results. But, safety by lack of knowledge: never!"

Whenever I remember Georges Köhler's life — with his family, in his laboratory, in his institute, in his collaborations with others, in the immunological community and in society — I am saddened and frustrated by the thought that he had to die so young, and much too soon.

Fritz Melchers

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Hot, fat and falling apart?

John C. Mutter

THE rifting of continental lithosphere, and subsequent generation of new oceanic lithosphere by sea-floor spreading in the resulting gap, occur almost continuously throughout the Earth's history. Nevertheless, it is possible that a critical suite of pre-conditions is required to initiate rifting, and that the process itself has defining attributes, which are not obvious from the record preserved in mature continental margins. Vast prisms of sediment typically cover continental margins, masking the structures that formed during the critical moments of continental breakup. Furthermore, many of the processes may be transients that leave little evidence in the ancient record. What we need are examples of the process in action. At any instant in the history of the Earth, however, active rifting is relatively rare, and the present is no exception. We have been faced with the problem that one of the most salient processes in the development of the Earth might evade our direct study. But on page 534 of this issue Taylor and others¹ report the remarkable properties of perhaps the only current example of the active rifting process — in the Woodlark basin off the shore of northeastern Papua New Guinea — and describe some unexpected findings.

Rupture

What are some of the outstanding questions that study of such an area might illuminate? One concerns the essence of the rifting phenomenon itself. Continental lithosphere, although perhaps less strong than was once thought, is not feeble and requires fairly large stresses to cause it to rupture. Nevertheless, there is ample evidence that it indeed ruptures and does so along huge lengths, forming continental margins that may be several thousand kilometres long. This presents something of a poser, in that it is not obvious that the Earth can produce the forces necessary to achieve this rupturing — at least not synchronously along an entire region of such great dimensions. How then does the rupturing occur? A likely mechanism involves propagation of stresses concentrated at a rift tip. This means that rupture occurs through a sort of progressive unzipping, rather than a synchronous rupturing along a great length of the lithosphere.

Evidence that continental lithosphere does indeed part in this way comes from the pattern of magnetic lineations immediately adjacent to margins. The oldest sequences of lineations are frequently seen to intersect the margin at an oblique angle and systematically age along the length of a margin, suggesting that the earliest phase of sea-floor spreading is

asynchronous². In the western Woodlark basin where Taylor *et al.* carried out their study of propagation, evidence for this process lies in the westward narrowing of the basin, associated with progressive termination of older sea-floor magnetic lineations. Propagation has progressed from east to west, beginning about 5 million years ago, and the active tip of the propagator at present lies immediately to the east of a string of extraordinary islands — the D'Entrecasteaux islands. What makes these islands special is that they include high-grade metamorphic rocks that have been rapidly exhumed from great depth in the continental lithosphere along shear zones, contemporaneously with the initiation and propagation of spreading. They therefore represent the youngest metamorphic core complexes on Earth³.

But propagation is far from simple. As Taylor *et al.* show, in some parts of the basin the westward extension of the locus of spreading is not achieved by propagation at all, in the sense of a progressive tearing apart of the lithosphere, but instead by the instantaneous development of a new spreading segment about 50 km long. Surprisingly, then, synchronous rifting does occur, but the dimensions of the rupture are restricted to relatively short regions. The maximum length over which rupture takes place synchronously may, in fact, be an indirect measure of the strength of the continental lithosphere. In other parts of the basin westward extension of the spreading axis involves propagation that is similar to that associated with propagation of spreading in purely oceanic settings, creating pseudo-faults at the boundary between continent and ocean⁴. Just what governs whether spreading advances by propagation or by synchronous initiation of a spreading segment is not resolved in Taylor and colleagues' study, but they have clearly shown that both modes are possible.

The surprises don't stop there. The widely held belief that major oceanic transform faults initiate at offsets in the geometry of rifted margins may not be appropriate to the development of spreading offsets along the Woodlark propagating ridge — no evidence is seen for the continuation of the ridge offsetting Moresby transform into the margin, and the offset seems to have begun life as a peculiar form of overlapping spreading centre in which continental crust is present in the overlap basin. Taylor *et al.* relate this odd behaviour to another surprising feature of the area: along the northern 'passive' margin seismic activity continues even after the spreading centre has been

active for around a million years, so deformation does not immediately localize into the plate boundary as soon as spreading commences. And the *pièce de résistance* is the unequivocal evidence that the spreading centre instantaneously re-oriented by about 5° through synchronous jumping along several hundred kilometres of the ridge some time in the very recent past. Rapid reorganizations of this type are exceedingly rare in mature spreading systems, and the clear evidence for a recent event here may represent the response to rapidly re-orienting stresses in breakup or an early spreading regime.

And to complete the line-up, Taylor and colleagues' study and work that we have reported elsewhere⁵ provide evidence in seismic reflection images for a distinct structure, dipping at a low angle to the north beneath the northern 'passive' margin, whose location coincides with that of an intermediate-magnitude earthquake. The earthquake, when analysed by Abers⁶ using waveform inversion, was found to indicate normal-sense motion on a low-angle detachment plane. Normal-sense motion on low-angle planes is difficult to achieve, given what we know about rock properties and the associated laws of friction, yet there is ample (if disputed) evidence in ancient extensional regions that such motion has occurred. The low-angle fault occurs at the very tip of the propagating system where the greatest concentration of stresses is likely to occur. Perhaps the unique environment of the rift tip promotes the conditions needed for this type of motion.

Are all of these features the strange inhabitants of a strange world and therefore little more than curiosities, or are they the common citizens of a realm, critical to our understanding of the Earth, that is seldom seen because it exists so fleetingly and is so easily buried? I believe that the latter is the case.

Weakness

But why then do they seem so unusual? To my mind, the answer might be that the lithosphere here, and, by implication, commonly associated with breakup, is extremely weak. Before extension began, the region suffered a prolonged period of plate convergence in which the crust was thickened by successive accretion events. Thick, hot crust is weak; so weak in fact that it might almost fall apart under its own weight⁷. Core complexes are thought to be the typical expression of deformation in hot thick crust, and there is no shortage of these features in the region. In addition to those on the islands, at least two are present on mainland Papua New Guinea and perhaps two others can be recognized in their geophysical signature north and east of the islands. Perhaps the other features that Taylor and colleagues describe are all the manifestations of

deformation in a very weak (or weakened) lithosphere — something that may be required for breakup to occur but cannot easily be deduced from the ancient record. In the Woodlark basin, perhaps uniquely, processes that are axiomatic to our understanding of the processes of rifting and the initiation of sea-floor spreading are active

today. As such, the region is destined to become a focal point for the study of these processes. □

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ASTRONOMY

Million-light-year galaxies and spectral forests

R. F. Carswell

SPECTRA of high-redshift quasars invariably show a veritable forest of absorption lines at wavelengths below that of the Lyman- α emission line arising in the quasar itself. These are due to hydrogen in gas clouds at redshifts below that of the quasar; corresponding heavy element lines are either weak or absent. Consequently, in the absence of other information, all one can learn about each of these systems is restricted to three quantities — the redshift, the integrated amount of hydrogen per unit area along a path through the cloud (the H I column density), and a measure of the internal velocity width. From these it is difficult to infer what one really wants to know, for example the heavy element abundances and conditions within the clouds, and where they occur. However, additional information is now becoming available. This month, a paper by Lanzetta *et al.* in *The Astrophysical Journal*¹ reports that many of the Ly- α systems at lower redshifts are associated with galaxies.

This clears up what had been one of the most important unresolved questions about the Ly- α absorption systems. A wealth of ideas for their origin had flourished in the absence of hard facts, including intergalactic clouds, intergalactic shocks, gravitationally bound systems involving cold dark matter and association with galaxies in some way. The absence of detectable heavy elements has occasionally been used as an argument against a galactic location, but this is not very compelling. The H I column densities for these systems are typically less than a thousandth of those corresponding to galaxies, and heavy elements would be detected only with great difficulty if we took 1/1,000 of the path through a galaxy. Another argument might be that the Ly- α systems are only weakly clustered on velocity scales of a few hundred kilometres per second, whereas galaxies are

strongly clustered. Again, this is not a strong point as galaxies in clusters may have had the outlying gas stripped away.

The most direct way of determining whether or not the Ly- α clouds are associated with galaxies is to see if there are galaxies at the redshifts of the Ly- α absorbers, which is what Lanzetta *et al.*¹ have now done. If the clouds are intergalactic, there should be no such association, whereas if galaxies tend to be found at similar redshifts then this provides clear evidence that many such systems lie in galaxies.

At the high redshifts where Ly- α is shifted into the optical window (redshift $z > 1.6$) this has not been done, mainly because the galaxies are so faint that detection of all but the brightest candidates is difficult, and measurement of their redshifts effectively out of the question. However, at low redshifts ($z < 0.5$) measurement of galaxy redshifts is possible. A key project using the Hubble Space Telescope has revealed a large number of Ly- α absorbers in this redshift range, and the new results from Lanzetta *et al.*¹ come from the determination of the redshifts of several galaxies in the fields of a number of these. The result is a clear association of the Ly- α systems with galaxies, with over a third of the Ly- α systems being identified this way. The lower limit arises from the fact that not all objects in the fields have measured redshifts. A correction for this suggests that about two-thirds of the Ly- α systems are associated with galaxies.

Identifying the galaxies responsible allows one to say much more, of course. The presence of absorption from some, and absence from others, allows Lanzetta *et al.* to estimate the sizes of the neutral hydrogen regions, and what fraction of the time a sightline through the galaxy will intercept enough neutral hydrogen to give rise to a detectable absorption line. The

typical diameter of galaxies measured this way is about 400 kpc (1.2 million light years), and almost all sightlines through the galaxy within this limit will give measurable absorption in neutral hydrogen.

The size estimate is an interesting one. The gaseous extent of normal galaxies is at least 15 times larger than the optical extent, and over four times greater than has been inferred from other ions. The presence of gas so far from the nucleus raises questions in itself. It is not clear if it is related to the inner regions or whether it arises as part of an accretion flow into (or ejecta from) the galaxy. These are questions which need further study.

The size estimate can be compared directly with that determined by other means. Over the years there have been several attempts to estimate the sizes of the Ly- α absorbers by looking for common absorption in close pairs of quasars. Most of these studies have been at higher redshifts, and generally they yield somewhat uncertain characteristic diameters in the range 100–800 kpc. One important recent paper², however, reports observations of a quasar pair with the Hubble Space Telescope, and a determination of a characteristic diameter of 400–450 kpc at redshifts $0.5 < z < 0.9$. This size estimate is in remarkably good agreement with that of Lanzetta *et al.*, and reinforces their galaxy identification result.

Important questions remain for further observational study. One is prompted by a similar search for galaxies corresponding to the Ly- α systems found in the Hubble Space Telescope spectra of the bright quasar 3C273³, which showed that most of the Ly- α absorbers in that case are not associated with luminous galaxies. The difference may be one of degree, as the Ly- α absorption lines in the 3C273 spectrum are considerably weaker than the ones used by Lanzetta *et al.* It might be that the systems with weaker lines are associated with fainter galaxies which would be missed.

A second point concerns the Ly- α systems at high redshifts. It appears that for redshifts up to about $z = 2$ the number of Ly- α absorbers per unit volume, corrected for the expansion of the Universe, is roughly constant. Above this redshift the numbers increase sharply with redshift, suggesting a rapidly evolving population. The reason for this change is not understood, but it could be that the excess at high redshift comes from intergalactic clouds which dissipate as the Universe expands. Testing this picture will be difficult, but suggestions that the clustering

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properties and intrinsic line widths for the Ly- α systems change with redshift could, if confirmed, reinforce it. More direct evidence, such as the small effect of the cloud dissipation on the absorption line shape, is expected to be difficult to measure.

The association of Ly- α systems with galaxies should renew the quest to detect heavy elements in them. It is likely that

even the outlying reaches of the galaxies suffer some heavy element enrichment from mixing in of supernova ejecta. Searches for lines from, say, carbon ions at high signal-to-noise ratio could be enlightening. □

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INFLAMMATION

A signal terminator

Nicolas G. Bazan

CELLULAR responses to inflammation involve the formation and accumulation of bioactive mediators, among which platelet-activating factor (PAF) is one of the most potent because it leads to cell damage by several mechanisms. An excess of PAF has been implicated in a variety of pathologies from ischaemia-reperfusion and necrotizing enterocolitis to asthma. The cycle of PAF synthesis and degradation (see figure) includes the hydrolysis of PAF by PAF acetylhydrolase, which converts the inflammatory mediator back to the biologically inactive lyso-PAF. The molecular cloning and *in vitro* expression of a human

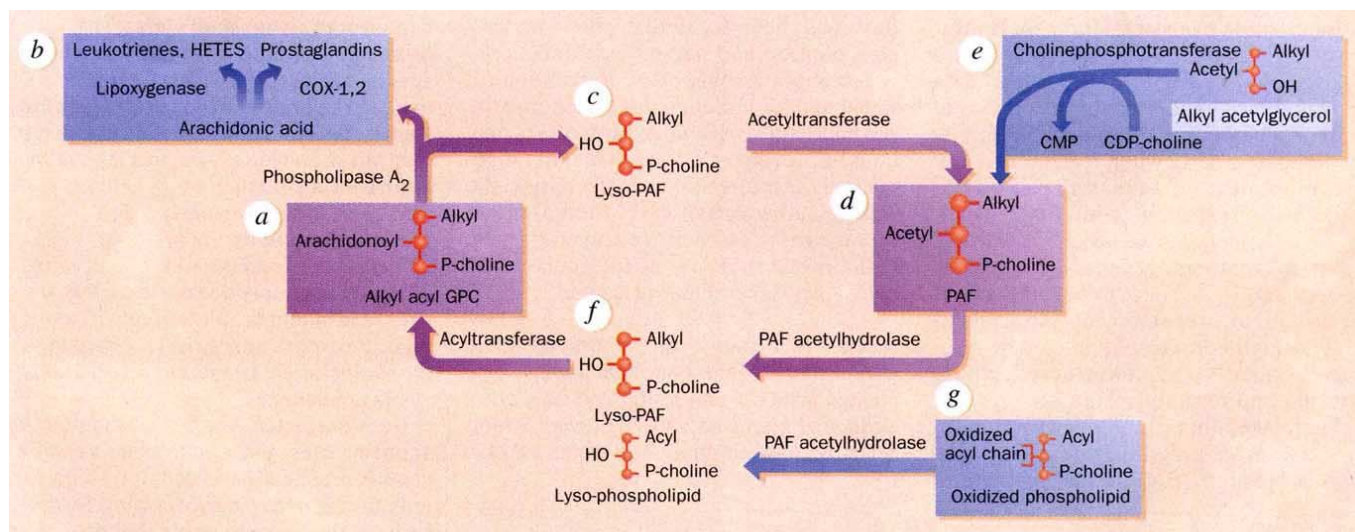
plasma PAF acetylhydrolase, reported by Tjoelker and colleagues on page 549 of this issue¹, open up the possibility of using the recombinant enzyme to terminate some types of inflammatory response. Indeed, the authors show that pretreatment with recombinant enzyme effectively blocks more than 80 per cent of the response in rat models of PAF-induced paw oedema and microvascular leakage in pleurisy.

PAF participates in normal cell function as well as in pathology. The dual nature of this bioactive phospholipid is similar to that displayed by glutamate, interleukin-1 and nitrous oxide. Synthesis of these

molecules occurs in cells in response to normal physiological stimuli, but when their concentrations increase above a certain level they can be harmful. PAF acetylhydrolase presumably provides a way of limiting excess PAF and its deleterious effects.

There is an intracellular² as well as a plasma^{3,4} form of the enzyme, and a regulatory subunit of this intracellular form has 99 per cent homology with the product of the *Lys-1* gene⁵; it is this gene that is defective in Miller-Dieker syndrome, a neurological disorder characterized by the absence of gyri and sulci in the cerebral cortex (lissencephaly). A defect in neuronal migration during brain development may underlie the smooth cerebrum of people afflicted with the syndrome, and PAF and an intracellular PAF acetylhydrolase may be involved in neuronal migration⁵.

When applied by a patch pipette into CA1 neurons (postsynaptic) of the hippocampus, PAF elicits glutamate release from Shaffer collateral terminals (presynaptic endings)⁶. Thus it may be a retrograde messenger in long-term potentiation, a cellular model of memory formation or plasticity. PAF also modulates interactions between leukocytes and endothelial cells⁷. In both of these cases, then, PAF is likely to be spatially restricted — in the brain to ensure that



again resulting in the removal of a potential exacerbator of inflammation. Events mediated by PAF include stimulation of the synthesis of leukotriene C₄, of PAF itself, and of prostaglandins. The prostaglandin effect is probably mediated by the PAF-induced COX-2 gene¹³. Metalloproteinases (collagenases)¹⁴ and heparin-binding EGF-like growth factor¹⁶ are also induced by PAF, as is activation of polymorphonuclear leukocytes (PMNs) and complement, and the formation of oxygen radicals. Oxidized phospholipids, which in a way are structural analogues of PAF because they have a fragmented fatty acyl chain in the C2 position, have many of the same effects as PAF, notably the modification of low-density lipoprotein, through which process they may participate in the early stages of atherosclerosis. Some or all of these events might be affected by exogenous plasma PAF acetylhydrolase. GPC, glycerol-3-phosphocholine; HETES, hydroxyeicosatetraenoic acids; CMP, cytidine monophosphate; CDP, cytidine diphosphate.

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only specific synapses receive signals, and in the blood vessels to prevent secretion into the bloodstream. If PAF were secreted into the bloodstream, generalized activation of leukocytes would result, causing 'downstream' organ damage (for instance in the lung). Plasma PAF acetylhydrolase is presumably a guardian that prevents the escape of PAF from its useful sphere of activity. Complementary DNA sequences provide strong evidence that this enzyme occupies a different location to that of the intracellular form, because the plasma form has a signal sequence¹.

During ischaemia and convulsions, phospholipase A₂ is activated and PAF⁸ accumulates in the brain along with free arachidonic acid⁹. In ischaemia-reperfusion brain damage, PAF antagonists are neuroprotective¹⁰. Moreover, membrane phospholipids can undergo oxidative damage to yield compounds that have structures with shorter peroxidized residues at their second carbon (see figure) and that mimic the action of PAF. In endothelial cells⁷ and brain¹¹, PAF acetylhydrolase catalyses the hydrolysis of these oxidized phospholipids and may be an important mechanism to prevent amplification of inflammation.

Recombinant PAF acetylhydrolase could be a therapeutic alternative to PAF antagonists, at least in some instances, because the enzyme may be able to break down PAF or PAF-like lipids at sites of inflammation, even when their concentrations are very high. Although the concept is exciting, as are the supporting data provided by Tjoelker *et al.*¹, it remains to be seen whether the approach is clinically viable, particularly when treatment is not begun until the inflammatory response is underway. Treatment with PAF acetylhydrolase may also represent a form of replacement therapy, especially in cases of acquired deficiency. Other uses might be found for recombinant intracellular PAF acetylhydrolase, if it can be produced similarly and delivered effectively into the appropriate cells.

There are other pathological situations in which PAF acetylhydrolase may provide protection. PAF is an activator of

immediate-early transcription factors¹², and of the inducible gene for prostaglandin synthase¹³. The PAF signal mediated via this inducible gene leads to increased prostaglandin synthesis, and in turn, the prostaglandins exacerbate inflammation. PAF also activates the expression of certain metalloproteinases that may degrade the extracellular matrix¹⁴. This genomic arm of signal transduction in inflammation may generate corneal ulcers in severe ocular inflammation and destroy cartilage in arthritis¹⁴. Moreover PAF is synergistic with the adhesion protein, P-selectin, in the induction of cytokine gene expression¹⁵ and heparin-binding EGF-like growth factor¹⁶ in human monocytes. So PAF, or related lipids, may be involved in the inflammatory and cell-proliferation aspects of atherosclerosis. In addition, PAF acetylhydrolase blocks the generation of modified low-density lipoprotein resulting from oxidative stress¹⁷: oxidized phospholipids have been implicated in this process, so protection presumably results from the hydrolysis of such lipids before they can modify proteins or signal inflammatory cells.

In experimental models, PAF reproduces many of the manifestations of asthma. An increased prevalence of severe asthma has been reported in children in a Japanese population that is deficient in PAF acetylhydrolase¹⁸, and elucidation of the molecular basis of this recessive trait will help to define the enzyme's physiological and pathological functions.

The rapid turning off of a pathological signal of the inflammatory response by recombinant PAF acetylhydrolase may limit or prevent cell damage and derangement of the intercellular matrix. Given the widespread effects of PAF, there is broad therapeutic potential in diverting the pathological PAF-way with Tjoelker and colleagues' recombinant enzyme. □

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DAEDALUS

Flash and bang

A LOT of subtle technology depends on unstable metal compounds. Silver halides, which decompose in light, are used in photographic film; silver and lead azides, which explode on heat or impact, are used to detonate explosives. Both, in effect, are chemical amplifiers. A film and a detonator both take a small amount of energy and use it to initiate a much larger chemical change. Daedalus is now combining the two.

He points out that lead and silver azides, and most other metallic detonators, are decomposed by light. There is even a photographic process based on such compounds, carefully diluted and dispersed to stop them exploding. But Daedalus has bolder photochemical plans. He wants them to explode. Intrepid DREADCO chemists are now preparing photographic emulsions, not of silver halide in gelatine, but of silver or lead azide in blasting gelatine. And instead of stabilizing them in an inert matrix, they are doping them with the most active sensitizing dyes known to photography. Their goal is to realize that old chemical joke, the explosive so sensitive that it goes off if you look at it. The faintest glimmer of light will set it off.

The immediate market for DREADCO's 'Photoexplosive Film' will be the printing industry. For this purpose, it will be coated onto blank metal plates. The printer merely loads the plate into a special camera, and points it at the image to be printed. When he presses the shutter, the emulsion explodes where the light hits it, and locally indents the metal. The result is an instant printing plate. A dark rinse to remove unexploded emulsion completes the process.

Photoexplosive Film will need careful design. If its energy density is too low, it will fail to indent the plate locally; if too high, the excess energy will propagate the explosion sideways and set off the whole emulsion.

Once perfected, however, it should soon find wider uses. Many metals can be shaped drastically and deeply by high energy-rate or explosive forming. So a heavy-duty photoexplosive process could forge a massive metal component instantly, directly from its drawing. On a small scale, a tiny sample of photoexplosive film would be the ultimate antitamper device for a container, a safe or a package. The tamperer would not only be badly shaken while automatically raising the alarm: he could be simultaneously photographed into the bargain. For the legitimate consignee, however, a reassuring bang when the package is opened will be the ultimate guarantee of its pristine condition.

David Jones

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Was HIV present in 1959?

Sir — A previously healthy 25-year-old man died of a mysterious illness in September 1959 in Manchester, UK. An autopsy performed by Dr George Williams¹ revealed the presence of *Pneumocystis carinii* and cytomegalovirus pneu-

monia as well as perioral and perianal herpes infections, but an underlying cause was not found. Following the clinical description of AIDS, the possibility that this man had AIDS was raised². With the advent of polymerase chain reaction (PCR) technology, the case was subsequently opened for re-examination.

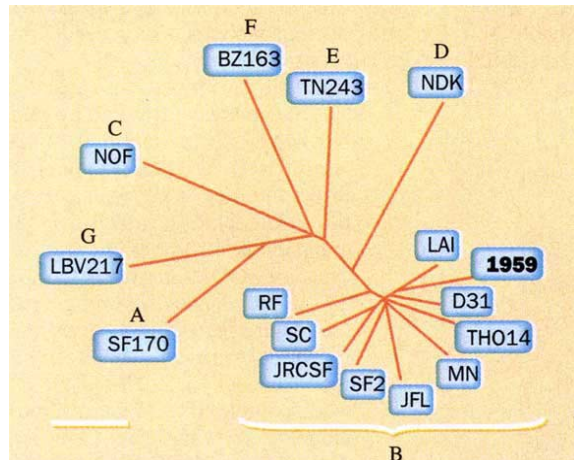
Paraffin-embedded tissues from the case, as well as tissues from a matched case of accidental death in 1959, were coded by Williams and submitted to Dr Gerald Corbitt (The Virology Unit, University of Manchester), who reported the detection of *gag* sequences of human immunodeficiency virus type 1 (HIV-1) by PCR in kidney, bone marrow, spleen and pharyngeal mucosa tissues, but not in liver or brain or in any tissues from the control case³.

Thus, it was concluded that this Manchester patient was the first documented case of AIDS in the medical literature. However, it remained unclear epidemiologically how he might have acquired HIV-1 infection. He was said to be a heterosexual who did not use intravenous drugs. He performed his National Service in the Royal Navy but was stationed exclusively in England, except for a brief voyage to Gibraltar in 1957.

Because the nucleotide sequence of the virus from a 1959 case of AIDS could shed light on the evolution of HIV-1 and related lentiviruses, we asked Corbitt for this information. His laboratory had determined the DNA sequence of two fragments of HIV-1 *env*, but was unsuccessful in obtaining more sequence data. We therefore offered to carry out a more extensive PCR and sequencing effort, as well as phylogenetic analysis. In November 1992, Corbitt sent us DNA extracted from kidney and bone marrow samples that had previously tested positive for HIV-1 (ref. 3).

In our hands, multiple attempts to amplify HIV-1 sequences by PCR from the bone marrow DNA were unsuccessful. However, we successfully amplified five fragments of HIV-1, covering the entire viral genome, from the kidney DNA. These PCR products were then cloned and sequenced. Sequence heterogeneity was observed among different clones, consistent with the presence of viral quasi-species typically seen in samples from infected people. Moreover, the short sequences of *env* obtained in Corbitt's laboratory closely matched our sequences for the corresponding region. All of our sequences from this case have been submitted to the HIV Database at Los Alamos National Laboratory and are available through Dr Gerald Myers (GenBank accession no. U23487).

The full-length nucleotide sequence was analysed independently by Myers of Los Alamos National Laboratory and Dr Eddie Holmes of Oxford University. Regardless of the region of genome examined, all phylogenetic analyses showed that the virus in the kidney sample was a member of HIV-1 clade B, the subtype currently prevalent in the United States and Europe (see figure). The branch length of the Manchester case was comparable to those found for recent HIV-1 clade B strains, suggesting that the 1959 sequence could not be distinguished from contemporary clade B sequences and thus raising the spectre of specimen contamination. Given the matched *env* sequences obtained in Manchester and New York plus the detection of quasiespecies with full-length genomes, the evidence suggests that any contamination was more likely to be by another clinical specimen than by cloned HIV-1 sequences or amplicons. We therefore decided to repeat the analyses



Phylogenetic relationship between the virus amplified from the kidney DNA (1959) and other clade B and non-clade B strains of HIV-1. The phylogenetic tree was constructed using the neighbour-joining method. Scale bar, 5%.

SUMMARY OF PCR RESULTS

Samples	β -globin	HIV-1 <i>gag</i>	HIV-1 <i>env</i>	HIV-2 LTR	HIV-1, 2/SIV <i>gag/pol</i>	HLA DQ- α
DNA from Corbitt (Nov 1992)						
1959/2 (kidney)	+	+	+	ND	ND	1.2, 4 (2, 3)
1959/8 (bone marrow)	+	—	—	ND	ND	1.2, 3
Tissue sections from Williams (Feb 1994)						
C (thyroid)	+	—	—	—	—	3, 4
13 (liver)	+	—	—	—	—	3, 4
5 (kidney)	+	—	—	—	—	3, 4
17 (kidney)	+	—	—	—	—	ND
14 (myocardium)	ND	—	—	—	—	ND
12 (pancreas)	+	—	—	—	—	ND
16 (mucosa)	+	—	—	—	—	ND
A (brain)	ND	—	—	—	—	ND
B (brain)	+	—	—	—	—	ND
Tissue sections from Corbitt (Feb 1994)						
K1 (kidney)	ND	—	—	—	—	ND
K2 (kidney)	+	—	—	—	—	ND
P (pharynx)	ND	—	—	—	—	ND
L (larynx)	ND	—	—	—	—	ND
B (bone marrow)	ND	—	—	—	—	ND

Detection of human β -globin gene was performed using outer primers GH20 and GH21 plus inner primers KM29 and KM38 or PC03 and PC04 as described by Saiki *et al.*⁴. Detection of HIV-1 *gag* was performed using outer primers P59 (1,366–1,389 of pNL4-3; 5'-GGACATCAAGCAGCCATGCAATG-3') and P52' (1,658–1,630; 5'-TTTGGTCTTGTCTTATGTCCAGAATGCT-3') plus inner primers P61 (1,395–1,428; 5'-AGAGACCATCAATGAGGAAGCTGC-3') and P52'2 (1,652–1,630; 5'-CCTGTGCTTATGTCCAGAATGCT-3'). Detection of HIV-1 *env* was performed using outer primer P5 (ref. 5) and PV3 (7,368–7,341; 5'-CAGTAGAAAAATTCCTCCACAATTAA-3') plus inner primers P7b (6,979–7,002; 5'-ATCAACTCAACTGCTGTTAAATGG-3') and PV3. Detection of HIV-2 LTR was performed using outer primers LTR A and LTR B plus inner primers LTR C and LTR D as described by Gao *et al.*⁵. Detection of HIV-1 and HIV-2/SIV *gag-pol* was performed using outer primers LenG11 (1,929–1,957 of SIVMM251; 5'-TATGTAGACAGATTCTACAAA-3') and LenP2 (3,395–3,371; 5'-CTATTAAGATATCATCCATGTACTG-3') plus inner primers LenG5 (2,016–2,035; 5'-AATGCTAACCCAGATTGCAA-3') and LenP4 (3,298–3,275; 5'-TGGTGACCCCTTCCATCCCTGTGG-3'). With some exceptions, PCR conditions were similar to those described in Zhu *et al.*⁵. Rare positive results for HIV-1 were noted, but they were shown to be contaminants by sequencing of the PCR products. LTR, long terminal repeat; ND, not done.

using another set of tissues from the 1959 case.

We contacted Williams and obtained tissue sections of brain, thyroid, kidney, pancreas, liver, myocardium and mucosa (probably pharynx) in February 1994. Concurrently, sections of kidney, pharynx, larynx and bone marrow were provided by Corbitt. DNA extraction and nested PCR were performed to detect LTR, *gag*, *pol* and *env* sequences of HIV-1 and HIV-2/SIV (simian immunodeficiency virus). Appropriate negative controls were included in each experiment, and positive controls were used to show that the PCR assays were sensitive (detection limit of about 2 copies) for all subtypes of HIV-1 (including group O) and HIV-2/SIV. As summarized in the table, PCR results were uniformly positive for β -globin in samples tested but negative for HIV-1 and HIV-2/SIV in all samples.

To investigate the apparent discrepancy, we performed tissue typing using the AmpliType HLA DQ-alpha PCR Amplification and Typing Kit (Perkin Elmer Cetus) to define the HLA DQ- α alleles in each DNA sample. As shown in the table, the first kidney DNA contained DQ- α -1.2 and -4 sequences, with traces of DQ- α -2 and -3, whereas the accompanying bone marrow sample contained DQ- α -1.2 and -3 sequences. In contrast, thyroid, liver and kidney samples provided directly by Williams contained sequences of DQ- α -3 and -4. These findings suggest that the collection of tissues examined was derived from at least two individuals. Although we cannot state with certainty which set of tissues is from the Manchester patient, the first set does appear to be more problematic in that a mixture of HLA DQ- α alleles was present. In our opinion, this finding invalidates the conclusion reached in the 1990 *Lancet* report³.

The results of our analyses reported here raise serious doubts about the authenticity of the 1959 Manchester man as the first documented case of AIDS due to HIV-1 infection. We suggest a further independent study of the remaining tissue samples to help resolve the matter, as this case is potentially important to our understanding of the evolution of primate immunodeficiency viruses.

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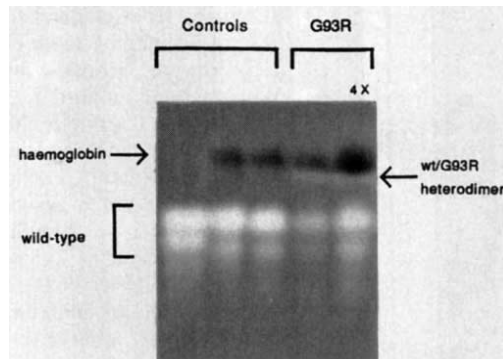
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A novel SOD mutant and ALS

SIR — No effective treatment exists for amyotrophic lateral sclerosis (ALS), a fatal degenerative disease characterized by the death of large motor neurons. About 5% of cases are familial, with the vast majority apparently occurring sporadically. The two forms are clinically indistinguishable and the underlying mechanisms may be related. The discovery of mutations in the SOD-1



Equal amounts (except 4 $\times$ and standard) of erythrocyte proteins separated on a native gel and stained for superoxide dismutase activity. Controls, left to right: CuZn SOD standard, normal human, spouse of proband. The assignment of wt/G 93R is based on comparison with other mutants we have made that have gained one positive charge⁷. Disease onset in the proband occurred at age 29 years. Family members had onsets at 36, 37 and 41 years leading to death within 12, 3 and 2 years, respectively. Solution assays for SOD-1 enzyme activity were determined directly on erythrocyte lysates by measuring loss of superoxide spectrophotometrically⁸. Many other assays are inhibited by haemoglobin which must be removed by precipitation with organic solvents. These solvents cause some precipitation of the less stable SOD-1 familial ALS mutants resulting in an underestimate of total activity (our unpublished results). Amounts of erythrocyte lysate for enzyme assay and for activity gels⁹ were standardized by assaying haemoglobin.

gene, encoding CuZn superoxide dismutase (SOD), in about 20% of familial ALS cases^{1,2} has thus stimulated efforts to understand the disease mechanism in these cases^{3,4}.

Two general mechanisms by which SOD-1 mutants cause disease have been proposed: loss of enzyme function, or the gain of an adverse function. Support for

the former comes from the finding that SOD-1 familial ALS patients, who are heterozygotes, have total enzyme levels reduced to 50–80 % of normal⁴. However, loss of enzyme activity could be incidental to the gain of a new adverse function. Strong evidence for such an adverse function comes from transgenic mice over-expressing SOD-1 familial ALS enzymes. These animals develop an ALS-like disease, yet the SOD enzyme activity in their central nervous systems is increased compared to controls^{3,4}.

Dominant negative mutations cause the loss of activity contributed by wild-type subunits in multimeric proteins (SOD is a homodimer) by direct interaction or by increasing the proteolytic turnover of the complex. After screening erythrocytes for SOD-1 enzyme activity in 27 UK families with familial ALS (see figure legend), we found one individual with only 30% of wild type levels ($17.3 \pm 0.93 \text{ U mg}^{-1}$), when compared to 71 control individuals, showing that we had detected a dominant negative effect. DNA sequencing¹ revealed that the SOD-1 familial ALS mutation responsible encodes a Gly 93 to Arg change (G93R)⁵.

To confirm the dominant negative nature of G93R we separated homodimers from heterodimer on a native polyacrylamide gel and stained for SOD activity (see figure). An equal interaction of native and mutant dimer interfaces would produce a 1:2:1 ratio of native homodimer to heterodimer to mutant homodimer. Comparison of the 4 $\times$ G93R lane with the 1 $\times$ controls indicates that an equal interaction is occurring and that approximately 25% of SOD molecules in the G93R erythrocytes are present as wild-type homodimers.

If G93R were completely dominant negative, only native homodimers should be active and enzyme levels would be 25% of control, rather than the 30% observed. Separation of the more positively charged wild-type/G93R heterodimer on the activity gel indicates that it has some residual activity which probably accounts for the additional 5% observed in the accurate solution assay. No activity corresponding to the G93R homodimer was observed. Thus, the activity gel confirms that G93R is a dominant negative mutation, although that it is not completely dominant negative in erythrocytes.

If the disease mechanism in SOD-1 familial ALS involves superoxide radicals, then increased severity of disease would be predicted for individuals with the G93R mutation, which is only 30% active, compared with the 50–80% activity of most mutants. In the family reported here

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the average age of onset was 36 years (see figure) compared to an average age of onset of 50 years for other SOD-1 patients with familial ALS⁶. This indicates that damage is occurring more rapidly in these individuals and suggests both that superoxide radicals are involved in the disease mechanism and that motor neurons are vulnerable to relatively small changes in the levels of SOD activity.

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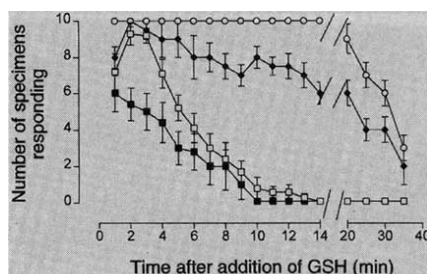
NO in hydra feeding response

SIR — In studying *Limulus polyphemus*, the horseshoe crab that has not evolved in 500 million years, Moncada and his group have observed, surprisingly, that the nitric oxide (NO) pathway may have been preserved throughout evolution¹. Recently, NO synthase has been reported to be present in different invertebrate groups². Here we have investigated whether the NO pathway is present in *Hydra* (phylum: Coelenterata), the most primitive invertebrate with a nervous system.

We have shown, using ³H-citrulline generation from ³H-L-arginine, that *Hydra vulgaris* express NO synthase activity. In hydra homogenates, ³H-citrulline production is dependent on the presence of NADPH and is significantly inhibited by preincubation with L-N^ω-nitroarginine methyl ester (L-NAME; 100 μM), a specific NO synthase inhibitor. Moreover, when resuspended in EGTA-Ca²⁺-free buffer, hydra homogenates incubated with 1 mM NADPH show a significant decrease ($P \leq 0.001$) in NO synthase activity, thus indicating that the NO synthase isoform is Ca²⁺-dependent.

By measuring nitrite (NO₂⁻), the breakdown product of NO, we find that hydra are able to release NO. Reduced glutathione (GSH; 2.5 μM), an activator of hydra feeding response (see below), enhances levels of NO₂⁻ (2–3-fold compared with basal levels; $P \leq 0.001$), as measured by the Griess reaction; this effect is abolished by L-NAME (100 μM).

We were astonished to find that NO is



GSH induces feeding response in *H. vulgaris*. GSH-induced response was analysed by recording the number of specimens showing the typical feeding response (tentacle writhing and mouth opening) each minute under a stereo-microscope. The response achieves its peak after a 2-min addition of GSH (2.5 μM) and disappears after about 10 min (controls) (□). A 24-h pretreatment with dbt²-cGMP (100 μM) (■) significantly shortened the GSH-induced feeding response. In contrast, a 1-h pretreatment of hydra by injecting L-NAME (100 μM) (○) or L-NMMA (100 μM) (◆) into the gastric cavity prolongs the GSH-induced feeding response by up to 40 min. Each point represents mean ± s.e.m. of 10 experiments. Two-way ANOVA test was used for significant differences between treatments. $P \leq 0.0001$ between controls and dbt²-cGMP or L-NAME or L-NMMA.

also involved in regulation of the hydra feeding response, which is the most primitive olfactory-like system present in a multicellular organism. The feeding response is a complex behavioural phenomenon consisting of tentacle writhing and mouth opening; it is elicited by the outflow of GSH from the prey itself when pierced by tentacle nematocysts³. The hydra feeding response arises a few seconds after GSH (2.5 μM) addition, reaches its peak within a very few minutes and gradually disappears in about 10 min, despite the presence of GSH in the bathing medium (see figure). The response is thought to terminate through the onset of an inhibitory mechanism⁴.

By using NO synthase inhibitors that reduce NO levels, we have demonstrated that NO is involved in the inhibition of the GSH-induced feeding response. In fact, when hydra were injected with L-NAME (100 μM) or L-N^G-monomethyl arginine into the gastric cavity and preincubated for 1 h, the GSH-induced feeding response was prolonged by up to 40 min (see figure); this effect was lost by co-incubation with L-arginine.

Furthermore, treatment of hydra with GSH (2.5 μM) induces an increase in cyclic GMP production, the maximum

effect being observed after a 1-min treatment (from 114 to 285 fmol; 10 specimens; $P \leq 0.001$). This increase is abolished by preincubation of hydra with L-NAME ($P \leq 0.01$), indicating that the cGMP generation results from NO release. The involvement of cGMP in the inhibitory mechanism of GSH-induced feeding response is demonstrated by a 24-h pretreatment with N²,2'-O-dibutyrylguanosine 3':5'-cyclic monophosphate (dbt²-cGMP; 100 μM), an analogue of cGMP, which can significantly shorten ($P \leq 0.0001$) GSH-induced feeding response in untreated hydra as well as in L-NAME- or L-NMMA-treated specimens. On the other hand, we have shown previously that the phosphodiesterase inhibitors theophylline and isobutyl methylxanthine dramatically inhibit the hydra GSH-induced feeding response⁵.

In conclusion, we confirm that the NO/cGMP pathway is preserved throughout evolution, in that it is involved in the most primitive model of an olfactory-like system present in a multicellular organism.

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MHC evolution

SIR — Peter Parham in News and Views¹ discussed results from Michael Brenner's group showing that CD1b, a molecule distantly related to MHC class I and class II molecules, binds a lipid antigen². One point he made (among others) was that mice do not have a CD1b-like molecule and that this lipid-binding function evolved, therefore, after the separation of the mammalian orders. Although this late evolution appears to have occurred with many MHC class I-like molecules, particularly those encoded in the MHC, it does not appear to be the case for CD1. A molecule homologous to CD1b has been identified in rabbits^{3,4} and similar molecules appear to exist in other species⁵. This indicates that CD1b-like genes evolved much earlier, before the human and rabbit lineages split, and that these genes were subsequently deleted from the mouse.

Interestingly, the murine CD1 locus on chromosome 3 (which contains two genes closely related to the human CD1d gene) spans a putative chromosomal translocation site that split chromosome 1 (which contains the human CD1 locus) during the divergence of humans and mice⁶. This location is the evolutionary equivalent of a 'smoking gun', strongly supporting the

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interpretation that CD1b and CD1b-like molecules were deleted during murine evolution. The conclusion that CD1b-like molecules are not a recent innovation is significant as it suggests that cell-mediated immunity directed at non-peptide antigens may have occurred early in the development of the immune system, perhaps even preceding the recognition of peptide antigens by the polymorphic, classical MHC class I and II molecules.

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'Functional' haplodiploidy

SIR — The coffee berry borer (*Hypothenemus hampei*) is the main insect pest of coffee worldwide. The recent evolution and rapid spread of high levels of resistance to endosulfan (a cyclodiene-type insecticide) in the South Pacific island of New Caledonia therefore constitutes a major threat to the international coffee industry¹. Like many other members of the scolytid bark beetle, this species shows a highly female-biased sex ratio and incestuous inbreeding²; thus, a single mated female enters the coffee berry and the numerous female progeny are obliged to mate with their few dwarf and flightless male sibs. In some bark beetle species this type of life history (inbreeding polygyny and spanandry) is associated with haplodiploidy².

We have previously shown that cyclodiene resistance in *H. hampei* is associated with a single point mutation in the GABA (γ -aminobutyric acid) receptor subunit gene *Resistance to dieldrin*, or *Rdl*, and that this same mutation is present in several strains from New Caledonia³. Previous insecticide bioassays have also shown that resistance is apparently sex-linked⁴. Thus, consistent with true haplodiploid inheritance, paternally derived resistance is not expressed in males or transmitted through males to progeny.

To test this possibility, we used two PCR-based diagnostic tests, polymerase chain reaction amplification of specific alleles (PASA)³ and single-stranded conformational polymorphism analysis (SSCP)⁵, to determine the genotype of the progeny of reciprocal crosses of F₁ males and females of known parentage. Interestingly, PASA with either the resistant or susceptible specific PCR primer (PASA-R or PASA-S) shows that both resistant and

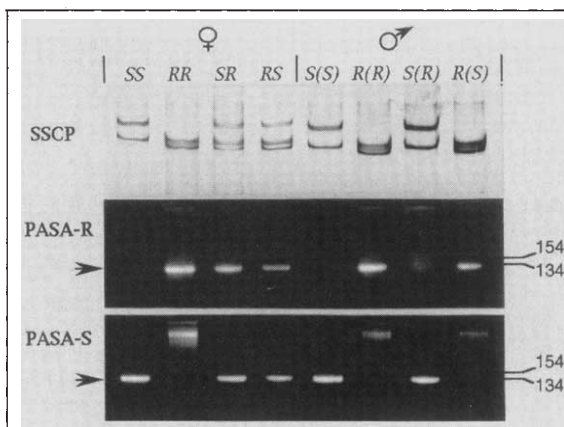


FIG. 1 SSCP and PASA analysis of males and females homozygous susceptible SS, homozygous resistant RR or heterozygous RS for resistance. Note that in females both maternal and paternal alleles (in parentheses) can be detected by either technique, whereas in male progeny both techniques fail to amplify paternally derived alleles. Thus, banding patterns associated with the predicted genotypes S(S) and S(R) are identical, as are the patterns for R(R) and R(S).

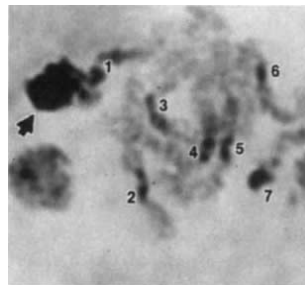


FIG. 2 Cytology of male spermatocyte. The arrow indicates the degenerating paternally derived set of chromosomes and the presumptive maternally derived set are numbered 1–7.

susceptible alleles fail to amplify in males when paternally derived. These data are confirmed by the simultaneous use of SSCP on the same amplification products: SSCP banding patterns for the S(S) and S(R), and the R(R) and R(S) each show the same pattern, because the paternally derived alleles (in parentheses) are not amplified in males (Fig. 1). This again appears consistent with haplodiploidy, in which males would be haploid and females diploid.

Cytological examination, however, reveals that although somatic tissues of females are $2n = 14$ as previously reported⁶, in the male soma one of the sets of chromosomes (presumably the paternal

set) is compacted into a darkly staining ball of chromatin. Further, in meiosis during the first (only) meiotic division the paternally derived set begins to degenerate while the maternally derived set condenses and divides (Fig. 2). Therefore, males are actually diploid, but the paternal set of chromosomes is condensed in both the soma and germ line, and is lost during sperm production. The coffee berry borer is therefore 'functionally' haplodiploid, although males still actually possess two sets of chromosomes.

Similar mechanisms for the destruction of paternally inherited genetic material have been observed in other insects and mites⁷. However, we believe our findings represent the first demonstration of the inheritance of insecticide resistance via functional haplodiploidy. This leads to an interesting mode of inheritance whereby females are continuously backcrossed to their female grandparents and may therefore explain

the observed rapid spread of resistance in New Caledonia^{8,9}: first, because recessive or semi-dominant resistance genes are exposed directly to selection in effectively hemizygous males; second, as there is no outbreeding, resistance will be perpetuated within individual, completely isolated, inbreeding lines; and third, within these lines, the continuous maternal backcrossing will promote the rate at which resistance genes achieve homozygosity. Thus, once a resistance-associated mutation has arisen it will be rapidly perpetuated within, and spread via, a single inbreeding line.

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Survival of the riskiest?

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Risk. By John Adams. UCL Press: 1995. Pp. 228. £35, \$65 (hbk); £12.95, \$21.95 (pbk). Distributed in the United States by Taylor and Francis.

THE torrent of books on risk shows no sign of abating, and for a very good reason; the topic is of considerable concern to all who live in modern, technologically based societies. There is abundant evidence from scientific reports and the daily press that technology has its dark side. But most of us are unwilling to withdraw to presumably safer low-technology environments, and the achievements of medical science have (so I believe) made us more interested in survival than any of our forebears ever were. We are all risk takers, and we hope that the emerging speciality of risk analysis will contribute to our safety. It is little wonder that risk analysis is described in this book as "the world's largest industry". Included as working in the industry are people employed in home safety, fire protection, casualty services, recreational safety, occupational safety, safety on the road, pure food and drug regulation, and the armed forces. The list goes on and on.

John Adams, a reader in geography at University College London, has published extensively in the field of road safety, the subject of his previous book *Risk and Freedom*. He now attempts to generalize the topic of road safety to a theory of risk in general. He provides useful comments on the work of such risk analysts as the US political scientist Aaron Wildavsky (*Searching for Safety*) and the German sociologist Ulrich Beck (*Risk Society*). He discusses a wide variety of risks and devotes a whole chapter to the greenhouse effect (he is sceptical about the evidence produced so far) but he is at his best when he writes about what he knows best: road safety.

Nearly one quarter of *Risk* is devoted to the analysis of the effectiveness of seat-belts and other automobile safety issues, for Adams has a great deal to say about them. His basic thesis is an extremely counterintuitive one: the mandatory use of seat-belts does not save lives among users and increases deaths among pedestrians.

The seat-belt debate seems to have been more heated in the United Kingdom than in the United States. In any event, Adams reports that both countries were "among the last of the world's highly motorized countries to implement seat belt laws". The largely unchallenged claim was made in the British parliament in 1979 that 1,000 lives and 10,000 injuries a year would be saved if seat-belts were used. In the United States, the claim was made in 1978 that 89,000 lives would be saved over the next ten years if seat-belts were used.

Adams explains these (to him, unjustified) claims by a widespread lack of awareness of "risk compensation and the possibility that there might be a behavioural response to the compulsory wearing of seat-belts". According to his interpretation, people wearing seat-belts feel more secure and so drive faster than they normally would. As evidence, he presents data from 17 countries containing more than 80 per cent of the world's car popula-



tion. During the early 1970s, road-accident deaths declined steeply in all countries (presumably because by then most cars were fitted with seat-belts) but declined most precipitously in countries without compulsory seat-belt legislation (presumably because in countries in which the wearing of seat-belts was compulsory, more people actually used their seat-belts and compensated by driving faster).

Adams's broad conclusion about seat-belt legislation is that because of risk compensation, many people drive faster while using seat-belts and accordingly increase their chances of a fatal accident; furthermore, because there are more speeding cars on the road, more pedestrians and cyclists are killed.

Here are examples of two common phenomena. First, legislation intended for one social purpose (saving the lives of motorists) has the unanticipated consequence of harming others (pedestrians and cyclists). Second, it is the educated and thoughtful people (drivers who drive carefully while seat-belted) who benefit

the most from social legislation (wearing a seat-belt reduces one's chances of being killed, if in a crash, by 41 per cent); it is the uneducated and the reckless who are harmed. The rich get richer and the poor get poorer.

An interesting parallel is a very successful US television show for children, 'Sesame Street'. It was started many years ago to level the playing field for children from culturally deprived homes. Audience research has shown, however, that because culturally favoured children are more likely than others to watch 'Sesame Street', the programme has actually widened the gap. Individual children, culturally deprived or not, of course benefit from watching it.

Adams's focus throughout the book — on statistics based on the risk perception of individuals as well as on the consequences of accidents for individuals — leads him to neglect one of the most promising avenues of research into risk: examining the formal properties of accidents themselves. To his credit, he describes the cultural, psychological and political contexts of risk behaviour, but he seems unaware of normal accident theory, as presented in Charles Perrow's *Normal Accidents* (Basic Books, 1984), which I reviewed in *Nature* (309, 185; 1984).

Perrow's normal accident theory concentrates on the properties of technological systems themselves rather than on the errors made by owners, designers and operators. Perrow thus seeks a more basic explanation for accidents than operator error or faulty equipment; he does this by analysing the ways in which the components of a system are interrelated. If Perrow were studying deaths from automobile accidents, he would undoubtedly want to examine each accident in a sample of accidents, looking for ways in which the driver, the automobile, other automobiles, speed, the road and other conditions are related. The outcome of wearing or not wearing a seat-belt would certainly be one situation examined.

It is unfortunate that Adams seems to be unaware of Perrow's pessimistic theory of normal accidents, but even without the inclusion of this perspective, his own pessimistic analysis is stimulating and rewarding. Both scholars would agree with the proposition that risk is endemic to a modern technological society, and living so as to limit one's exposure to danger is the best strategy for the thoughtful individual to follow. Fasten your seat-belt and drive slowly. □

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The evolution of life's complexity

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The Major Transitions in Evolution. By John Maynard Smith and Eörs Szathmáry. W. H. Freeman: 1995. Pp. 346. £16.99, \$29.95 (pbk).

I HAVE a fondness for big themes in biology, and this book more than delivers. It spans the major transitions in evolution, starting with a prebiotic mix of free molecules and ending with the evolution of human language (from primaeval soup to alphabet soup). This is a grand and daunting sweep, and it is to the authors' credit that they have carried it off. I suspect many readers will approach the book, as I did, with caution and a dash of scepticism, but all in all it is a splendid and rewarding *tour de force*. Everyone knows John Maynard Smith as a master of evolutionary biology, and Eörs Szathmáry has a special interest in the early stages of biological evolution. They both think in terms of models, mathematical and otherwise, yet this book is written for the non-mathematician. Readers should have a background in basic biology and evolution, but need no advanced knowledge.

Increase in complexity

Before going any further, let me reveal the plot. Unlike a detective story, there is no danger of my spoiling it for the reader; in fact, my comments should have the opposite effect of encouraging the reader to get past superficialities and find out what the authors themselves have to say. The major transitions considered are all enormous steps towards an increase in complexity, and the authors make it admirably clear right from the start that they are not talking about progress, that forbidden concept so often wrapped in mysticism.

In the opening section on the origin of life, the authors sensibly summarize many of the prevailing views while not hesitating to expose their own biases. This is a good review for people who do not often think about the subject. We soon get to the central matter of primitive replication, starting with Spiegelman's famous experiments on the test-tube replication and selection of RNA, hypercycles and other models designed to illuminate the early stages of the evolution of life. The appearance of RNA, DNA and proteins is treated in some depth, with an extensive discussion of the origin of the genetic code and translation. Protocells come next: there is a need for compartments with membranes that can not only keep the insides and the outsides separate but also selectively transport some key mol-

ecules in or out. These compartments lead to the problem of how metabolism with its plethora of enzymes might have arisen, and to the formation of the double membranes of prokaryotes and the origin of chromosomes.

The first known fossil bacteria originated 3,500 million years ago, whereas the first eukaryotes did not arise until 1,500 million years ago. The enormous time delay serves to emphasize that the emergence of eukaryotes is a major transition. It involves the creation of mitosis, a more elaborate organization of the genome and the appearance of organelles such as centrioles and cilia. The authors lean heavily (and quite properly) on the ideas of Tim Cavalier-Smith and examine in a level-headed way the possible role of symbiosis in these early events. This leads to a good discussion of the origin of sex and meiosis and the nature of species. Here the authors grapple with the difficult problem of what 'species' means in the prokaryotic world. (I remember raising this point some years ago with Robert MacArthur who, after thinking about it, finally said, "Well, I guess that's why I work on birds".) Next comes a splendid account of a topic of great current interest: intra-genomic conflict. How do the different parts of the genome and the various replicating organelles in a cell compete with one another for reproductive success, and what are the consequences? From this the authors leap to symbiosis between different organisms, looking at how such associations might have evolved and the possible benefits and the costs.

The next step is to multicellular organisms and the origin of differentiation. (I have some qualms about this section and will return to it.) This is followed by excellent, brief accounts of gene regulation and cell heredity and of development and pattern formation.

A major transition is the appearance of animal societies, raising all the familiar but important ideas about kin selection and its possible role in the origin of societies. Although these genetic aspects of sociality are emphasized, the authors are quite aware that there are other selective reasons why animals group together in societies. One subject they cover is the division of labour in societies, a matter they touch on earlier when tackling the specialization of enzymes, and of cells in cell differentiation during development. Insect societies have beautiful examples in which labour is divided by having a mixture of workers of different ages doing different sets of tasks; and, of course, the more familiar case in which the castes are morphological, with each morph specializing in particular tasks. At this point the authors quickly turn to human societies and suddenly we are surrounded by Rousseau, Plato, Durkheim and Adam Smith and told about the

palaeontology of early humans.

All this is preparation for the final big fling — the invention of human language. In a fascinating last chapter, the authors provide a good introduction to basic Chomskyan theory, acknowledging the help of David Bickerton's *Language and Species* (1990). One comes away imagining that one understands what grammar is and one gains a better understanding of what humans can do that other primates cannot. Our linguistic ability, the product of our huge brains, is in the authors' view the ultimate peak in the evolution of living complexity.

Critics of books that attempt to cover so much ground usually like best what they know least about; as soon as they venture into their own particular territory they rapidly become less enthusiastic. So all I say now must be seen in the light of this law of 'ignorance varying inversely with criticism'. The difficulty is inevitable if an entire book is written from one vantage-point. But the alternative — a collection of essays by different specialists — is likely to please only other specialists who just read the chapter closest to their own interests. Here we have a bold attempt to link the interests of many people in one cohesive story, and I applaud the result. Nevertheless, I cannot resist a strong urge to add my bit.

In their preface, the authors thank Jonathan Cooke for a "splendid two-day tutorial", the result of which is a first-rate chapter on developmental biology. My regret is that they did not spend two days with me before they attacked the origin of multicellularity. My list of examples would have been both different and richer. Although I too would have included *Volvox* as a case study for the origin of differentiation, it is a pity that the authors missed out all its lovely genetics. The idea of using budding yeast, a complex ascomycete that arose late in evolution, as a prototype for the problem of early differentiation seems unfortunate, although the discussion of the genetics of its mating-type differentiation is interesting. One final complaint about this chapter: I would have dragged in complex ciliates with their elaborate and fascinating differentiation, all within one cell membrane.

Division of labour

This leads me to an old hobbyhorse of mine which also concerns the division of labour, both at the cell level and between organisms in animal societies. I think that there is, at all levels, a correlation between an increase in size and an increase in the division of labour, and that this is a fact of tremendous significance; I certainly would have included it.

Another missed opportunity where I would love to have rushed in is in the discussion of the evolution of insect societies. Instead of considering only the advanced

examples of societies among insects such as bees, ants and termites with huge colonies and multitudes of sterile workers, I would have also dwelt on the beautiful work on primitive social wasps that have only a few individuals in a colony, perhaps just a group of potential queens that come together in a simple primitive nest. These small wasp colonies have provided a wonderful testing ground for models of how insect societies might have arisen, and have produced exciting recent discoveries. It is exactly what is needed in a chapter on the rise of animal societies.

Finally, I wish the authors had concluded with a grand comparison of the different steps of increasing complexity. They do this a bit at the beginning and now and then along the way, but I would have added something at the very end for which they seem to have perfectly set the stage: a small section on comparative transitionology. But this is their book, not mine. They have looked at the great panorama of biological evolution from the point of view of theoretical biologists, and the end result is both rewarding and fascinating. □

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The Boyle we have lost

P. M. Rattansi

Robert Boyle by Himself and His Friends. Edited by Michael Hunter. *Pickering and Chatto*: 1994. Pp. 188. £49.95.

ROBERT Boyle, according to John Evelyn the diarist, had become almost a national monument in his later years. "There was no man whose Conversation was more Universally sought after, courted, & cultivated, by persons of the highest rank & quality: Princes, Ambassadors, Forrainers, Scholars, Travellers, & Virtuosi. . . So as one who had not seen Mr Boyle was look'd-on as missing one of the most valuable Objects of our Nation: It was in his Philosophical Apartment, Tapissered & furnish'd with Instruments for Trials & natural Experiments. . . that he often Entertain'd those who came to visit him."

Soon after Boyle's death in 1692, there were plans to publish a comprehensive biography, offering "this bright example" of a life spent selflessly in the study of God's works to a "degenerate age", where "Lewdness and Irreligion" were rampant. As Michael Hunter shows in the fascinating introduction to his collection of texts, all these attempts came to naught. Bishop Burnet (well known for his *History of My*

Own Time) had kept notes on his conversations with Boyle for a period extending over three decades. He was able to use them with great effect in the funeral sermon he preached on Boyle's death. That sermon, according to Hunter, established the image of Boyle that we have inherited. Thomas Birch conveyed to posterity his standard edition of the works of Boyle, published in five folio volumes in 1744 and in a revised six-volume edition in 1772.

Burnet himself was unable to implement his intention of writing a fuller biography. He eventually secured the interest of a young protégé, William Wotton (1666–1726), whom Swift was to cast as the champion of the Moderns in *The Battle of Books* (1704). Wotton's plan was ambitious and would have resulted in the first real intellectual biography in English, had it been realized. But the sheer bulk of the material defeated Wotton. Wotton had confided to Evelyn his wish not merely to describe Boyle's scientific discoveries but to compare them with "what has bin since raised upon his Foundation". This involved contending with more than 20 scientific works published by Boyle in his lifetime, besides others on morals and theology, as well as the many manuscript tracts and a huge correspondence to which Boyle's executors had given him access. The long and hitherto unpublished 'chapter' from Wotton's uncompleted biography in the present volume illustrates his difficulties. It deals with Boyle's air-pump experiments. Once Wotton had summarized the tedious detail in Boyle's "scrupulous" account, added comments on the more recent ones by the Newtonians, Keill and Hawksbee, and discussed the implications of Newtonian attraction for the "vacuist" explanations Boyle had offered, he had written no less than 40,000 words on a single if important set of discoveries. It is perhaps hardly surprising that he abandoned the project even before disgrace for "sundry indecencies" forced him to give up his ecclesiastical living at Milton Keynes and take refuge in Wales.

Hunter seeks to persuade us that "The Boyle we have Lost" re-emerges from the shade as we study the material he has painstakingly reassembled from manuscript sources. How much of the material is really new? "An Account of Philaretus", an autobiographical account of the first 16 years of Boyle's life, was printed by Birch and the full text made accessible by R. E. W. Maddison in his *Life of Boyle* in 1956.

Burnet's funeral sermon on Boyle was printed soon after its delivery. The Evelyn letters have been available in Bray's edition of Evelyn's *Diary and Correspondence* (1850–52). Three of the substantial documents are therefore well known. Of the other material, the most important is the Burnet memorandum for the new light it casts on various aspects of Boyle. Nearly half of it describes Boyle's various encounters with others who claimed to have learnt alchemical secrets by summoning supernatural and demonic aid. Hunter has already discussed in print their possible bearings on Boyle's view of magic and alchemy. He also prints the notes pre-

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Robert Boyle — bright example for a degenerate age.

pared by Sir Peter Pett to assist Burnet. They dwell in part on Boyle's practical interests, played down in Birch, but at the same time discourage any simplistic reading of Boyle as champion of the post-Restoration mercantilist state. The long Wotton chapter is a disappointment, after raising hopes of a new kind of history. When it is omitted from the count, only a third of the documents can be said to offer material for a fuller, if not wholly novel, view of Boyle.

Hunter has nevertheless placed us in his debt by making available in a convenient format the primary biographical sources of information on Boyle. His introduction to the documents teases out a great deal that is new and the volume is a suitable curtain-raiser for his larger Boyle project, which is to include all of Boyle's published works (12 volumes) as well as a far more complete edition of his correspondence than in Birch's standard edition. □

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Home and away

Ashley Montagu

The Story of a Marriage: The Letters of Bronislaw Malinowski and Elsie Masson. Volume 1: 1916–20. Volume 2: 1920–1935. Edited by Helena Wayne. Routledge: 1995. Pp. 196/261. Each volume £40, \$65 (hbk); £13.99, \$17.95 (pbk).

WE owe these remarkable letters between two remarkable people — Bronislaw Malinowski, the founder of social anthropology and modern field work, and his wife Elsie Masson — to the energy and persistence of their youngest daughter Helena. It was Helena who travelled to Mexico to find her father's papers, which had been taken there in 1946, and she has now arranged both the letters and the diary material with skill. Here for the first time we are introduced to the delightful and talented Elsie. Indeed, the letters display Malinowski as her admiring sensitive friend and devoted lover. Not that Malinowski, with his normal distribution of faults, was ever in any sense forbidding; but he was often away from Elsie after returning to England in April 1920. This separation, and the worry about the impending birth of their first child, at which he could not be present, not to mention very limited funds, meant that some people might not have seen him at his best.

They arrived in England shortly after the First World War, "the war to end all wars", when homes were promised "fit for heroes to live in". But with many of the surviving war heroes standing in the gutter selling pencils or eking out an existence as pavement artists along with the tragic figures of the unemployed, it was clear that something was very wrong with the country. Nevertheless Elsie and Bronio faced the austerity of the postwar years with equanimity: they remained cheerful, encouraged and inspired each other and never faltered in their loyalty and love.

In those years, the general psychosis cast its shadow of depression and cynicism over everyone. The feeling was one of betrayal, of abandonment, as if one had been emptied of all that one had formerly believed to be the principles of civilized life. In reaction to the dismaying bitterness and helplessness was the artificial gaiety of the 1920s and 1930s. Thrust into the midst of this bittersweet environment, with its warm enthusiasm for the new sciences as well as the arts, music and literature, the

young Malinowskis found themselves very much alive.

In 1922 Malinowski began teaching at the London School of Economics (LSE), where two years later he established the first department of anthropology in the United Kingdom. He became part of the Bloomsbury circle of writers, artists and intellectuals of which it was said that almost every couple was a triangle and practically everyone lived in squares. It was a time when a surprising number of bright young people frequented the dark unfathomed caves at LSE, when the devotion that existed between Malinowski and his wife seemed hardly credible.

During his 19 months of fieldwork studying the Trobriander islanders between 1914 and 1918, Malinowski kept a strictly personal diary, written in Polish

leagues and students. His derisive manner would sometimes give offence, when in fact he was simply trying to be amusing. All of this gave a rather skewed view of Malinowski, and many were incapable of seeing him from any other angle. Yet the diary is probably one of the most scrupulously honest self-criticisms ever penned by a man of feeling, who with all his flaws was at once an intellectual, a scientist, an original thinker, a superb teacher and a delightful human being.

The best corrective to any ill-considered view of Malinowski the man are the letters between him and Elsie from 1916 to her early death from multiple sclerosis at the age of 45 in 1935. *The Story of a Marriage* is a unique love story, chronicling their meeting, courtship and marriage. With the spread of Malinowski's fame and his long

absences from home, Elsie was often left missing him badly. She never complained, for she understood that the hustle-bustle of a life led as an itinerant sophist was necessary to Malinowski; but her poignant letters to him cry out with her ache for them to be together.

Malinowski was not insensitive to this. In a letter to her as early as May 1926, he wrote: "I know that there will be a long gap in my correspondence... and I feel very unhappy about it. I have also an access of strong feeling of homesickness, after you and the babies and feel I am being cheated out of so much in life by not being able to live more

with them. . . I cannot go away again from you after having been at home for about six weeks only!" But he continued to go away for weeks and sometimes months at a time, doing research in Africa and the Americas. Elsie supported and encouraged him in all his wanderings, gently enabling him to perceive the folly of his occasional intemperateness. Her high intelligence and winsome character shined out from the beginning of their marriage and throughout the ten years of the progressively debilitating course of her illness.

The Story of a Marriage contains much of interest about friends, colleagues, students, notable scientists, Malinowski's academic activities and travels, and all the information necessary for an all-round assessment of the man and the scientist. It will be of great value to social anthropologists and their students, to historians of science and to future biographers. But equally important is the introduction it gives to Malinowski's wife, a woman of extraordinary character and sensibility who deserves to be widely known. □

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Malinowski in the Trobriand Islands, from his *The Sexual Life of Savages* (1929).

and never intended for anyone's eyes but his own. Nevertheless, 25 years after his death it was edited by his second wife and translated and published in 1967 as *A Diary in the Strict Sense of the Term*. There is hardly a page where Malinowski does not write with deep feeling of "E. R. M.", his spiritually betrothed Elsie Rosaline Masson; long after their marriage in 1919, Malinowski wrote that she "had treasures to give and the miraculous power to absolve sins".

Malinowski was something of an icon to his anthropological colleagues, as well as to other social scientists. With publication of his magnificent *Argonauts of the Western Pacific* in 1922, his reputation was at once established as a major star in the constellation of anthropologists, a reputation continually reinforced by his many subsequent publications. His diary therefore came as something of a shock, revealing his preoccupation with sex, his references to the Trobriander islanders as "niggers", and his depressions, illnesses and pills. And the role of "Peck's bad boy" that he would now and then choose to play, his ornery sense of humour and his occasional hapless asperities did not sit well with some of his col-

Mary Evans

Polyadenylation of *c-mos* mRNA as a control point in *Xenopus* meiotic maturation

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***c-mos* protein, encoded by a proto-oncogene, is essential for the meiotic maturation of frog oocytes. Polyadenylation of *c-mos* messenger RNA is shown here to be a pivotal regulatory step in meiotic maturation. Maturation is prevented by selective amputation of polyadenylation signals from *c-mos* mRNA. Injection of a prosthetic RNA, which restores *c-mos* polyadenylation signals by base pairing to the amputated mRNA, rescues maturation and can stimulate translation in *trans*. Prosthetic RNAs may provide a general strategy by which to alter patterns of mRNA expression *in vivo*.**

THE *c-mos* proto-oncogene encodes a serine-threonine kinase that has been strongly implicated in the control of vertebrate meiosis and the early embryonic cell cycle¹. In *Xenopus*, over-expression of *c-mos* protein causes precocious meiotic maturation and prevents mitotic cleavage after fertilization²⁻⁴. Female mice lacking a functional *c-mos* gene display reduced fertility due to parthenogenetic activation of the cell cycle, accompanied by ovarian cysts and teratomas^{5,6}. Although aberrant expression of *c-mos* in somatic cells can cause transformation, *c-mos* messenger RNA is normally found only in the germ line, and is present in the unfertilized egg. In frogs, translation of *c-mos* mRNA is essential for progression through meiosis I and extrusion of the first polar body^{2,7}.

Cytoplasmic poly(A) addition is correlated with the activation of many maternal mRNAs in a broad range of species, and can stimulate translation during oocyte maturation and early development⁸⁻¹⁰. It is controlled by specific sequences in the 3' untranslated region (3'UTR). These include the highly conserved sequence AAUAAA, and nearby U-rich sequences that have been designated cytoplasmic polyadenylation elements (CPEs)^{8,10}. Sequence inspection shows that, the *Xenopus c-mos* 3'UTR contains such elements¹¹. Based on these findings, we proposed that cytoplasmic polyadenylation of *c-mos* mRNA might stimulate translation of *c-mos* protein and therefore be critical to the onset of meiosis (Fig. 1)^{8,11,12}. Consistent with this view, *Xenopus c-mos* mRNA receives poly(A) during maturation¹². Moreover, the *c-mos* 3'UTR, linked to a reporter, stimulates translation in a manner that requires polyadenylation¹². Here we demonstrate that polyadenylation of *c-mos* mRNA is indeed a pivotal step in controlling the initiation of meiosis, and not merely a peripheral manifestation of it. Our results establish a novel approach using *trans*-acting RNA prostheses to affect mRNA expression *in vivo*.

Amputation of 3'UTR prevents maturation

If polyadenylation of *c-mos* mRNA is critical for the onset of maturation (Fig. 1), then oocytes containing *c-mos* mRNA that cannot be polyadenylated should fail to mature. To test this prediction, *Xenopus* oocytes were injected with antisense DNA oligonucleotides complementary to segments of the *c-mos* 3'UTR that lie 126 or 883 nucleotides upstream of the poly(A) tail. Upon annealing to the mRNA, these antisense oligonucleo-

tides (designated anti-126 and anti-883) should cause digestion of the RNA strand of the RNA-DNA duplex by RNaseH present in the oocyte cytoplasm. In this fashion, the polyadenylation signals of *c-mos* mRNA, including both AAUAAA and the CPE, should be severed from the bulk of the mRNA, preventing cytoplasmic polyadenylation (Fig. 2a). To assay maturation, we monitored the appearance of a white spot at the animal pole of the oocyte, indicative of germinal vesicle (nuclear) breakdown (GVBD) and the completion of first meiosis.

Injection of either the anti-126 and anti-883 oligonucleotides inhibits oocyte maturation in response to progesterone (Fig. 2). Injection of the sense strand of either oligonucleotide sequence (Fig. 2b) has little or no effect on maturation. These data have been obtained in 20 experiments, using two different chemical syntheses of the oligonucleotides.

c-mos mRNA in oocytes injected with antisense oligonucleotides is amputated in its 3'UTR, as expected, and is stable (Fig. 2c). RNAs prepared from oocytes that had been injected with sense or antisense oligonucleotides and then treated with progesterone were analysed by northern blotting. Oocytes injected with the sense strand of either oligonucleotide contain mRNA that is full length (Fig. 2c, lanes 1 and 5). This is true both in the oocytes that did mature in response to progesterone (Fig. 2c, lanes 1 and 5), and in those few that did not (Fig. 2c, lanes 2 and 6). In contrast, after injection of the antisense oligonucleotides, oocytes that did not mature contained *c-mos* mRNAs that had been shortened by the expected length (Fig. 2c, lanes 4 and 7). The few oocytes that matured in spite of injection of the antisense oligonucleotide contained intact, full-length *c-mos* mRNA (Fig. 2c, lane 3), further reinforcing the conclusion that removal of the end of the *c-mos* 3'UTR prevents maturation.

Injection of DNA oligonucleotides can have nonspecific effects in oocytes, most notably a general inhibition of protein synthesis.¹³ To circumvent this potential complication, each oligonucleotide was purified by a combination of gel electrophoresis and HPLC. As a result, none of the oligonucleotides reduces the level of protein synthesis, as measured either by incorporation of ³⁵S-methionine into total protein, or by the production of luciferase protein upon co-injection of luciferase mRNA (not shown). Moreover, oocytes injected with antisense oligonucleotides mature in response to low doses of injected *c-mos* mRNA (see below), attesting to their viability and to the specificity of the inhibition. We therefore conclude that the terminal portion of the *c-mos* 3'UTR is necessary for oocytes to mature in

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FIG. 1. Proposed role of polyadenylation of *Xenopus c-mos* mRNA in the control of meiosis. *Xenopus c-mos* mRNA is inefficiently translated in resting oocytes^{1,7}. Cytoplasmic polyadenylation of *c-mos* mRNA, caused by progesterone addition, is proposed^{8,11,12} to stimulate *c-mos* translation, thereby elevating the level of *c-mos* protein. (Changes in the rate of *c-mos* proteolysis (not depicted) may also contribute to the rise in *c-mos* protein levels after progesterone treatment³².) The accumulation

of newly synthesized protein leads to completion of meiotic maturation, including activation of maturation promoting factor (MPF), breakdown of the germinal vesicle or nucleus (GVBD), and the appearance of a white spot at the animal pole of the oocyte. Proposed roles of *c-mos* protein in second meiosis and in metaphase arrest before fertilization are not depicted.

response to progesterone, and that it controls the translation of the mRNA rather than its stability.

3'UTR segment stimulates translation

c-mos protein appears early in maturation, before GVBD^{2,7}. Thus, if the model in Fig. 1 is correct, the *c-mos* 3'UTR should receive poly(A) and stimulate translation before GVBD. To test this prediction, oocytes were injected with chimeric mRNAs in which the last 321 nucleotides of the *c-mos* 3'UTR were

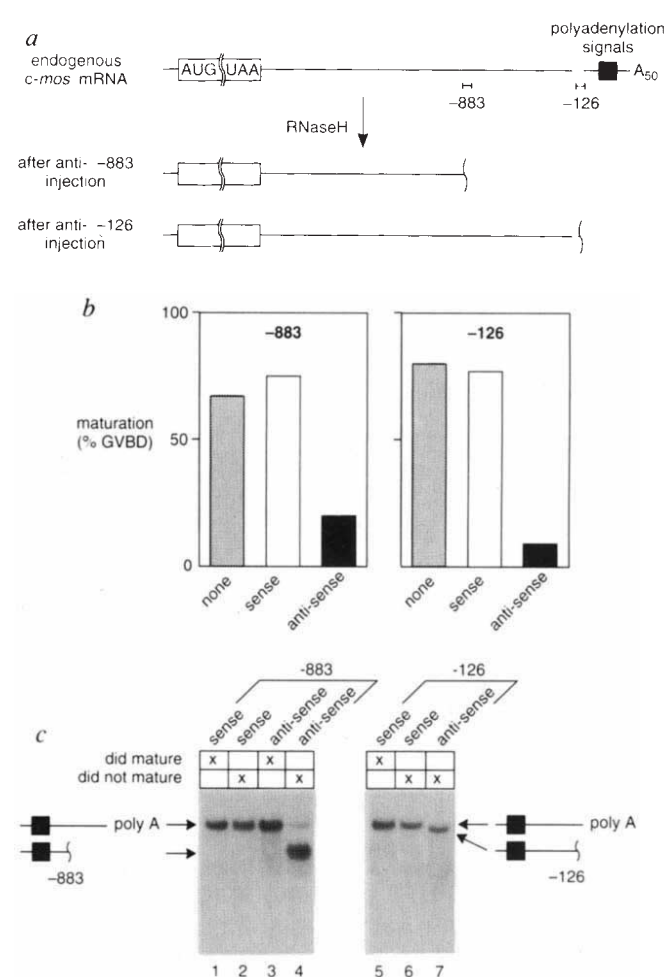
joined to a translational reporter, luciferase, the activity of which could be assayed readily. Translational activity was assessed at various times after progesterone addition (Fig. 3). An mRNA carrying a portion of the wild-type 3'UTR, including its polyadenylation signals, stimulated translation four- to fivefold by GVBD₅₀ (mRNA 1, Fig. 3), the time when half of the oocytes display a white spot. Translational stimulation was detectable before any oocytes had undergone GVBD. In contrast, translation of an identical mRNA that lacked the

FIG. 2. Injection of antisense oligonucleotides inhibits maturation by amputating the 3'UTR from the coding region of *c-mos* mRNA. **a**, Schematic diagram of *c-mos* mRNA illustrating the sites to which the antisense oligonucleotides hybridize, relative to the open reading frame (open box), polyadenylation signals (filled box) and poly(A) tail. The polyadenylation signals include both AAUAAA and the CPE. The antisense oligonucleotides anneal to sequences located 126 or 883 nucleotides upstream of the polyadenylation site of *c-mos* mRNA. Below are shown the predicted products resulting from RNaseH digestion after injection of the antisense oligonucleotides into oocytes. **b**, Injection of anti-126 and anti-883 oligonucleotides inhibits meiotic maturation. Oocytes were injected with sense and antisense versions of oligonucleotides-883 or -126, and were then exposed to progesterone. The height of the vertical bar indicates the percentage of the cells that had matured (that is, resumed and completed first meiosis), as assayed by the appearance of white spots at the animal pole. **c**, *c-mos* mRNA from cells injected with antisense oligonucleotides lacks its 3'UTR, but is stable. The indicated sense or antisense oligonucleotides (lanes 1 and 2, oligonucleotide-883S; lanes 3 and 4, oligonucleotide-883A; lanes 5 and 6, oligonucleotide-126S; lane 7, oligonucleotide-126A) were injected and progesterone added. RNA was prepared from oocytes that matured (lanes 1, 3, 5) or did not mature (lanes 2, 4, 6, 7) after progesterone treatment, and was analysed by northern blotting. Filled box, coding region of *c-mos* mRNA.

METHODS. For **b**, oligonucleotides used for injection were as follows. The numerical designations indicate the positions relative to the nucleotide to which poly(A) is added, designated -1; S or A indicates antisense or sense orientation relative to *c-mos* mRNA.

-883S 5'-ATCTAGTACAGTATCTCAATGTCCA-3' 25-mer position -883 to -859
 -883A 5'-TGGACATTGAGATACTGTACTAGAT-3' 25-mer position -859 to -883
 -126S 5'-GCACTGAAATACAAGCAAGGATATG-3' 26-mer position -126 to -101
 -126A 5'-CATATCTCTGCTGTATTTTCAGTGC-3' 26-mer position -101 to -126.

The oligonucleotides were purified by denaturing polyacrylamide gel electrophoresis followed by HPLC. Manually defolliculated oocytes were injected with 100 ng of oligonucleotide and then incubated in progesterone (10 $\mu\text{g ml}^{-1}$). Maturation was scored at a time twice that of GVBD₅₀ (defined as the time when half of the oocytes had undergone GVBD), in this and all subsequent experiments, unless noted otherwise. In most experiments, absence or presence of a germinal vesicle was checked by fixation in 5% trichloroacetic acid followed by manual dissection. Maturation was assayed 'blind', without knowledge of how the cells had been treated. The values obtained represent the average of 15 (the -126 oligonucleotides) and 5 (the -883 oligonucleotides) separate experiments. In each experiment, oocytes from a different frog were used and each oligonucleotide was injected into at least 30 oocytes. **c**, RNAs were isolated from mature and non-mature cells that were injected with sense or antisense oligonucleotides. RNAs were isolated by homogenization in 50 mM Tris pH 7.9, 5 mM EDTA, 2% SDS and



300 mM NaCl, followed by phenol/chloroform extraction and ethanol precipitation. RNA samples from an equivalent number of cells were denatured with glyoxal and resolved on a 1% agarose gel. Blotting and hybridization were done as described¹². The RNA hybridization probe was complementary to positions -1,850 to -1,746 of the *c-mos* 3'UTR, adjacent to the translational termination codon (position -1,951).

FIG. 3 The 3'UTR of *c-mos* mRNA stimulates translation at or before nuclear breakdown. *a*, A schematic diagram of the chimeric luciferase/*c-mos* mRNAs used. mRNA 1 contains 1,680 nucleotides of the luciferase gene followed by the last 321 nucleotides of the wild-type *c-mos* 3'UTR, including its polyadenylation signals (filled box). The polyadenylation signals include both AAUAAA and a CPE. mRNA 2 is identical, except that it lacks the last 83 nucleotides of the 3'UTR, which contain the polyadenylation signals. *b*, The wild-type *c-mos* 3'UTR stimulated translation before GVBD₅₀. Manually defolliculated oocytes were injected with 5 ng of the mRNA indicated. Half of the injected cells were incubated in progesterone (10 $\mu\text{g ml}^{-1}$) and the other half were not. At various times thereafter, extracts were prepared and luciferase activity measured as described¹². The ratio of luciferase activity present in matured (+P) versus non-matured (-P) oocytes is plotted against the time after progesterone addition. Filled circles, mRNA 1; open circles, mRNA 2. GVBD₅₀ is indicated by an arrow. Data were obtained from at least 20 oocytes per time point. By injecting mRNAs and analysing luciferase activity at GVBD₅₀, we have obtained similar results in six mRNA 1 and three mRNA 2 experiments.

METHODS. *a*, mRNAs were prepared by transcription of the pLuc/*c-mos* plasmid using T7 RNA polymerase¹², after cleavage with either *Xho*I (for mRNA 1) or *Dra*I (for mRNA 2). The *Dra*I cleavage site is located 61 nucleotides upstream of the AAUAAA sequence. -P no progesterone added, +P progesterone added.

last 83 nucleotides of the 3'UTR, including the region removed by the anti-126 oligonucleotide, was not stimulated during maturation (mRNA 2, Fig. 3). From these results, we conclude that the 3'UTR of *c-mos* stimulates translation at or before GVBD occurs.

Rescue by synthetic *c-mos* mRNA

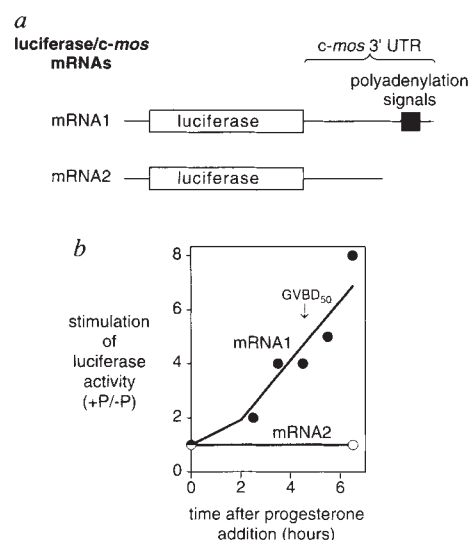
If oocytes carrying amputated *c-mos* 3'UTRs fail to mature because they are unable to polyadenylate and translationally activate *c-mos* mRNA, then their ability to mature should be rescued by injecting *c-mos* mRNA containing polyadenylation signals. To test this prediction, oocytes that had first been injected with the anti-126 oligonucleotide were subsequently injected with functional *c-mos* mRNA prepared by transcription *in vitro*. The synthetic mRNA comprised the 5'UTR and open reading frame of *c-mos* mRNA joined to the last 83 nucleotides of its 3'UTR (mRNA 3, Fig. 4*a*). This synthetic mRNA lacks the region complementary to the antisense DNA oligonucleotide and so cannot be affected by its presence. (In addition, injected oligonucleotides are degraded rapidly in the oocyte cytoplasm.) As expected, maturation is blocked again by the antisense oligonucleotide (Fig. 4*b*). Importantly, the ability of these oocytes to mature in response to progesterone is restored by subsequent injection of the synthetic mRNA carrying polyadenylation signals (mRNA 3, Fig. 4*b*). Rescue is specific and dependent on polyadenylation signals, as it is much less effective with a mutant *c-mos* mRNA from which the polyadenylation signals have been deleted (mRNA 4, Fig. 4*b*).

Rescue of maturation by *c-mos* mRNA is dose dependent, increasing throughout a tenfold range of RNA concentration (Fig. 4*c*). The mRNA lacking the *c-mos* polyadenylation signals (mRNA 4) rescues at least tenfold less efficiently than does the mRNA containing them (mRNA 3, Fig. 4*c*). This is consistent with the observation that the *c-mos* 3'UTR stimulates translation of a reporter mRNA six- to tenfold (Fig. 3 and ref. 12).

Previous work has demonstrated that injection of large amounts of *c-mos* mRNA induces maturation even in the absence of progesterone treatment². The mRNA injected in our experiments does not do so (Fig. 4*b*), presumably because of the dose dependence demonstrated in Fig. 4*c*: the amount of mRNA injected in our experiments (30 pg) is 100- to 1,000-fold lower than in the earlier work (ref. 2).

Prosthetic RNAs rescue maturation

Artificial and natural antisense RNAs can be used to control gene expression at multiple levels¹⁴⁻¹⁶. Typically, antisense RNAs



basepair to an RNA target and thereby prevent its function, either by steric interference or by causing the target's destruction. To test the involvement of *c-mos* polyadenylation in maturation, we developed a strategy in which a *trans*-acting RNA, by base pairing to its cellular target, could supply polyadenylation signals and thereby activate the mRNA target's translation *in trans*.

The strategy of the experiment is shown in Fig. 5*a*. Oocytes containing amputated *c-mos* mRNA, generated as described above, were subsequently injected with an RNA prosthesis designed specifically to reattach polyadenylation signals to the amputated mRNA, and thereby rescue its translation activity. This prosthetic RNA contains, at its 5' end, a sequence complementary to the last 54 nucleotides of *c-mos* mRNA left after amputation by the anti-126 oligonucleotide, followed by the last 83 nucleotides of the natural *c-mos* 3'UTR, including the signals for cytoplasmic polyadenylation and translational activation (Fig. 3). If, after annealing to amputated *c-mos* mRNA, the signals in the prosthetic RNA still cause polyadenylation, then they should cause translational activation. Thus injection of the prosthetic RNA should enable oocytes containing the amputated mRNA to mature in response to progesterone.

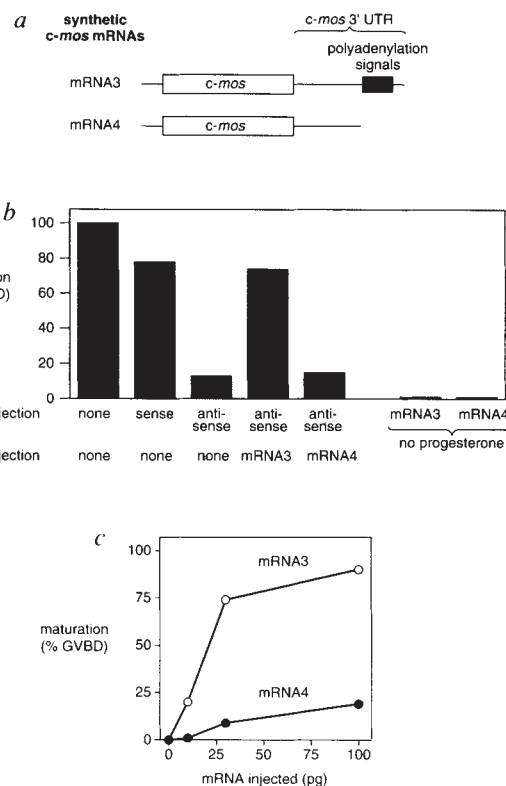
The prosthetic RNA rescues maturation (Fig. 5*b*). Oocytes were first injected with the anti-126 oligonucleotide, which reduced the fraction of oocytes that matured in response to progesterone to 6%, as compared to 90% without any injection (Fig. 5*b*). Subsequent injection of the prosthetic RNA rescued maturation, such that 58% of the oocytes injected with the prosthesis matured. Injection of buffer did not rescue maturation.

Variant prosthetic RNAs lacking cytoplasmic polyadenylation signals (Fig. 5*c*, RNA 1), or lacking complementarity to the amputated *c-mos* mRNA (Fig. 5*c*, RNA 2), did not rescue maturation. In addition, rescue was dependent on the amount of prosthetic RNA injected (not shown). Thus restoration of the function of the amputated mRNA, namely the ability of the oocyte to mature in response to progesterone, required that the prosthesis be able to base-pair, carry cytoplasmic polyadenylation signals, and be present at sufficient concentration in the oocyte.

To determine whether the small RNAs received poly(A) during maturation, radiolabelled prosthetic RNAs and variants thereof, as depicted in Fig. 5*c*, were prepared by transcription *in vitro* in the presence of ³²P-UTP and injected into oocytes. The oocytes were then treated with progesterone, and polyadenylation of the injected RNAs was assayed by electrophoresis and autoradiography. RNAs that carried polyadenylation sig-

FIG. 4 Rescue by synthetic *c-mos* mRNA. **a**, Schematic diagram of synthetic *c-mos* mRNAs with or without *c-mos* polyadenylation signals. mRNA 3 contains the *c-mos* coding region and 5'UTR joined to the last 83 nucleotides of the *c-mos* 3'UTR, including its polyadenylation signals, AAUAAA and a CPE (filled box). mRNA 4 is identical, except that it lacks the last 40 nucleotides of 3'UTR, which contain the polyadenylation signals. **b**, Injection of synthetic *c-mos* mRNA after amputation of *c-mos* mRNA rescues maturation. Oocytes were first injected with the anti-126 oligonucleotide (-126A; bar 3) or with the equivalent sense strand oligonucleotide (-126S; bar 2). After 1 h, oocytes that had been injected with the anti-126 oligonucleotide were injected a second time, with either mRNA 3 or mRNA 4 (bars 4 and 5). All oocytes were then exposed to progesterone. As a control, mRNAs 3 and 4 were injected into oocytes that were not subsequently treated with progesterone; neither mRNA induced maturation (bars 6 and 7). The heights of the vertical bars indicate the percentage of the cells that matured, determined by the appearance of a white spot at the animal pole. **c**, Rescue of maturation by synthetic *c-mos* mRNA is dose dependent. Manually defolliculated oocytes were first injected with 100 ng of the anti-126 oligonucleotide (-126A). After 1 h, the same oocytes were injected with varying amounts of mRNA 3 (open circles) or mRNA 4 (filled circles). Maturation was initiated by the addition of progesterone of up to $10 \mu\text{g ml}^{-1}$. The percentage of cells that mature (display a white spot) is plotted against the amount of mRNA in the second injection.

METHODS. **a**, Synthetic *c-mos* RNAs (mRNAs 3 and 4) were generated from the pGEMc-*mos*/3'UTR plasmid. pGEMc-*mos*/3'UTR was constructed by cloning the *SacI* fragment containing the *c-mos* coding region from pXmos-8 into the *SacI* site of pGEM3Z to create pGEMXmos-8^{2,7}. The 5' end of *c-mos* cDNA was inserted using synthetic oligonucleotides into the *EcoRI* and *SacII* sites of pGEMXmos-8, generating pGEMc-*mos*^{2,7}. The *c-mos* 3'UTR was added to the coding region by cloning the *PstI* (blunted with T4 DNA polymerase) *XbaI* fragment from pGEM-83/+2 *c-mos* into the *BamHI* (blunted with T4 DNA polymerase) *XbaI* sites of pGEMc-*mos*, generating pGEMc-*mos*/3'UTR¹². This plasmid contains the normal *c-mos* 5'UTR that begins at the major transcriptional start site (50 nucleotides upstream of the AUG start codon), the natural context of the AUG start codon, the entire *c-mos* coding region, 203 nucleotides of the *c-mos* 3'UTR immediately downstream of the translation stop codon followed by the last 83 nucleotides of the *c-mos* 3'UTR including the signals for maturation-specific poly(A) addition. mRNA 3, containing the *c-mos* 5'UTR, coding region, and that portion of the 3'UTR present in pGEMc-*mos*/3'UTR, was transcribed by T7 RNA polymerase from *XbaI*-cleaved pGEMc-*mos*/3'UTR¹². mRNA 4, transcribed by T7 RNA polymerase from *NdeI*-cleaved pGEMc-*mos*/3'UTR, is identical except that it ends 41 nucleotides before the poly(A) tail, and so lacks signals required for polyadenylation. **b**, Manually defolliculated oocytes were injected with 100 ng of oligonucleotide,



either -126S or -126A (see Fig. 2 legend for sequences). A portion of the oocytes injected with -126A were then injected a second time with 30 pg of mRNA 3 or mRNA 4. Maturation was initiated by adding progesterone ($10 \mu\text{g ml}^{-1}$) to the injected cells. To test whether the synthetic *c-mos* mRNAs were capable of maturing oocytes independent of the addition of progesterone, oocytes that had not been injected with any oligonucleotides were injected with 30 pg of either mRNA 3 or mRNA 4. Cells from these injections were not exposed to progesterone. The results represent the average of two separate experiments. In each, oocytes from a different frog were used and each sample was injected into at least 30 oocytes.

nals received poly(A) during maturation, whether or not they contained sequences complementary to the end of amputated *c-mos* mRNA (Fig. 5d, lane 1 and 2, 5 and 6). The variant RNA lacking polyadenylation signals was inactive (Fig. 5d, lanes 3 and 4). These results demonstrate that polyadenylation signals function in the prosthetic RNAs, and strongly suggest that they can rescue maturation only when attached to *c-mos* mRNA by base pairing.

Prosthetic RNA stimulates translation

To test directly whether a prosthetic RNA carrying polyadenylation signals stimulates translation (in addition to restoring the capacity to mature), we performed the experiment in Fig. 6. An mRNA was prepared by *in vitro* transcription that contained a luciferase open reading frame followed by a portion of the *c-mos* 3'UTR, ending at a similar site to mRNAs that had been amputated with the anti-126 oligonucleotide. The translation of this mRNA (mRNA 2) after injection, as monitored by luciferase activity, is unaffected by progesterone treatment (Fig. 6b). This was expected because it lacks the signals necessary for polyadenylation during maturation. In contrast, oocytes injected with a mixture of this mRNA and the prosthetic RNA exhibited a fivefold stimulation of translation during oocyte maturation (Fig. 6b). An intact mRNA carrying the last 321 nucleotides of the *c-mos* 3'UTR, including its polyadenylation signals, stimu-

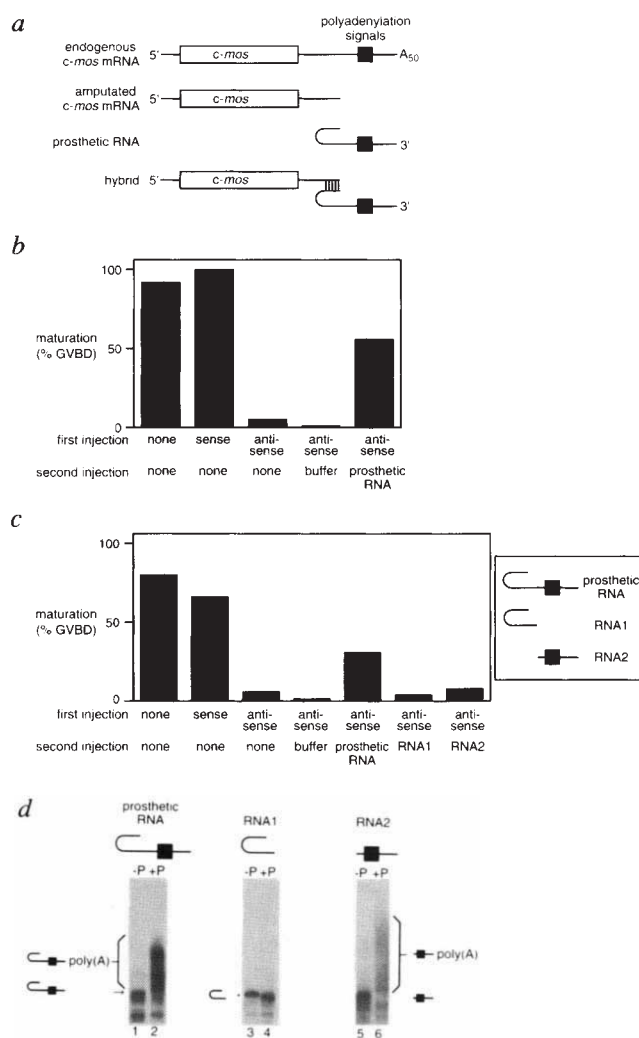
lated translation 13-fold (mRNA 1, Fig. 6b). (The level of stimulation seen with mRNA 1 is greater here than in Fig. 3 because the oocytes were incubated for a longer time before assaying luciferase accumulation¹².) From these results we conclude that translation can be stimulated *in trans* by an RNA carrying cytoplasmic polyadenylation signals.

Polyadenylation as a control point

Our data strongly suggest that polyadenylation of *c-mos* mRNA is an integral part of the regulatory cascade that initiates meiotic maturation, rather than a secondary consequence of it. *c-mos* protein is probably required to generate active maturation-promoting factor (MPF), a complex of p34^{cdc2} kinase and cyclin that is critical in cell cycle control^{1,33}. If so, it follows that polyadenylation of *c-mos* mRNA should occur independently of, and before, activation of MPF. The mechanism by which polyadenylation of *c-mos* mRNA is stimulated may involve activation of a 'core polyadenylation apparatus' that acts on many different mRNAs and contains an RNA binding factor and a cytoplasmic poly(A) polymerase¹⁷⁻¹⁹. Alternatively, factors that are specific for *c-mos* mRNA may be activated. Because cytoplasmic polyadenylation also stimulates the translation of cyclin A1, B1 and B2 mRNAs¹², and cyclins are required to form MPF, polyadenylation's involvement in the control of maturation is likely to extend beyond *c-mos*.

FIG. 5 Prosthetic RNA rescues maturation. **a**, Experimental strategy. The prosthetic RNA was designed to hybridize to the 3' end of endogenous *c-mos* mRNA after it had been amputated by injection of the anti-126 oligonucleotide, and thereby re-attach polyadenylation signals. The polyadenylation signals (filled box) include both AAUAAA and a CPE. **b**, The prosthetic RNA rescues maturation. Oocytes first were injected with either the antisense oligonucleotide (-126A, bar 3), the equivalent sense strand oligonucleotide (bar 2), or were not injected (bar 1). After 1 h, oocytes that had first been injected with the antisense oligonucleotide received a second injection of either buffer (bar 4) or prosthetic RNA (16 ng; bar 5). Oocytes were then exposed to progesterone. The height of the vertical bar indicates the percentage of the cells that matured (displayed white spots). **c**, Complementarity and polyadenylation signals are required for efficient rescue. Oocytes were injected with oligonucleotides alone or oligonucleotides followed by injection of prosthetic RNA, or variant prosthetic RNAs, designated RNA 1 or RNA 2 and shown on the right. All oocytes were exposed to progesterone. The heights of the vertical bars indicate the percentage of the cells that matured. **d**, Prosthetic RNA receives poly(A) during maturation. Radiolabelled RNAs were injected into oocytes and the oocytes incubated in the presence or the absence of progesterone, as indicated above each lane. RNA was extracted from separate single oocytes and analysed by denaturing gel electrophoresis. The position of polyadenylated RNAs, where present, are indicated by brackets.

METHODS. In **b**, the prosthetic RNA contains 159 nucleotides: 54 nucleotides of complementarity to *c-mos* 3'UTR, followed by 83 nucleotides of the *c-mos* 3'UTR sequence, plus vector sequences at its 5' end and between the region of complementarity and the 3'UTR sequence. Prosthetic RNA is generated from the plasmid, pGEM/*c-mos*/prosthetic. An insert containing 54 nucleotides of sequence complementary to positions -196 to -143 of the 3'UTR of the *c-mos* mRNA was created by annealing two single-stranded DNA oligonucleotides. The region of complementarity was designed to hybridize just upstream of positions -126 to -101 in the 3'UTR of *c-mos* mRNA, the extreme 3' end of the 3'UTR left after amputation by injection of the antisense oligonucleotide, -126A. The insert containing the region of complementarity was cloned into the *Hind*III and *Pst*I sites of pGEM-83/+2 *c-mos*¹² creating pGEM/*c-mos*/prosthetic. In this plasmid the region of complementarity is positioned upstream of the last 83 nucleotides of the *c-mos* 3'UTR including the signals necessary for maturation specific poly(A) addition. To generate prosthetic RNA, pGEM/*c-mos*/prosthetic DNA was cleaved with *Xba*I and transcribed *in vitro* using SP6 RNA polymerase and the 5' cap analogue ApppG¹¹. Manually defolliculated oocytes were injected with 100 ng of oligonucleotide, either -126S or -126A. A portion of the oocytes injected with -126A were then injected a second time with buffer, or with 16 ng of prosthetic RNA. After injection, maturation was initiated by adding 10 μ g ml⁻¹ progesterone to the injected cells and assayed as in Fig. 2. **c**, Manually defolliculated oocytes were injected with 100 ng of oligonucleotide, either -126S or -126A. A portion of the oocytes injected with -126A were then injected a second time with buffer alone, or with 16 ng of either the prosthetic RNA, RNA 1 or RNA 2. RNA 1 is generated by *in vitro* transcription with SP6 RNA polymerase of *Nde*I-cleaved pGEM/*c-mos*/prosthetic. RNA 1 (118 nucleotides) contains 54 nucleotides complementary to the *c-mos* 3'UTR followed by 43 nucleotides of the *c-mos* 3'UTR (nucleotides -83 to -41), but lacking the signals required for maturation-specific poly(A) addition. The remainder of the RNA is composed of vector sequence of the 5' end and between the complementarity and 3'UTR. RNA 2 (referred to elsewhere as -83/+2 *c-mos* RNA¹²) was generated by *in vitro* transcription



with SP6 RNA polymerase of *Xba*I-cleaved pGEM-83/+2 *c-mos*. RNA 2 (113 nucleotides) contains 29 nucleotides of vector sequence followed by 83 nucleotide of the *c-mos* 3'UTR including its polyadenylation signals (filled box)^{11,12}. After injection, maturation was initiated by adding progesterone to the injected cells. **d**, Radiolabelled prosthetic RNA, RNA 1 and RNA 2 were generated by *in vitro* transcription as above¹¹. The RNAs were injected into manually defolliculated oocytes and maturation was initiated by adding progesterone (10 μ g ml⁻¹) to the injected cells. After maturation, RNAs were isolated from injected cells and analysed by electrophoresis through 8% denaturing polyacrylamide gels¹¹. Rescue by the prosthetic RNA was repeated six times, in experiments using 20 to 30 oocytes per sample. Results with RNA 1 and RNA 2 were obtained twice and once, respectively, in the same experiments.

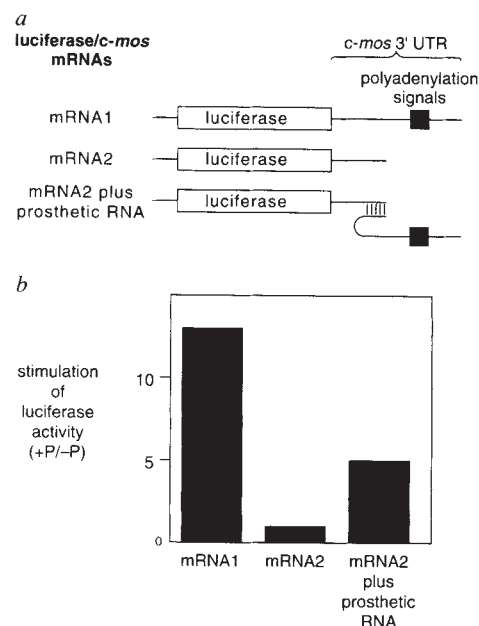
Female mice lacking a functional *c-mos* gene produce oocytes that complete first meiosis, although more slowly than in wild-type animals^{5,6}. This, together with the results reported here and previous work¹⁻⁴, implies that the regulation of meiosis differs in mice and *Xenopus*. As suggested elsewhere³³, as frogs produce large numbers of matured oocytes synchronously and mice do not, there may be different mechanisms of induction in the two species. Consistent with this view, protein synthesis is required for the progression of first meiosis in frogs but not mice^{1,20,21}. The translation of *c-mos* mRNA, stimulated by cytoplasmic polyadenylation, is likely to be one such requisite step. The synthesis of new cyclin proteins²², which is also likely to be stimulated by cytoplasmic polyadenylation¹², may also be critical.

The experiments reported here identify a biological process in which cytoplasmic polyadenylation probably plays a key regulatory role. Polyadenylation may be a general mechanism used to activate a battery of mRNAs during early development⁸. *bicoid* mRNA, which is important in establishing the anterior-posterior axis in *Drosophila*, and *fem-3* mRNA, a regulator of sex determination in *C. elegans*, may be controlled in this fashion, because changes in their poly(A) length are associated with changes in their translational activity^{23,24}.

Trans-acting prosthetic RNAs

The use of prosthetic RNAs to stimulate translation of an endogenous mRNA suggests new opportunities for intervention into

FIG. 6 Prosthetic RNA stimulate translation. *a*, Schematic diagram of experimental strategy and of the RNAs used. The mRNAs are the same as in Fig. 3. By design, the 3' end of mRNA 2 is very close to the 3' end of the endogenous *c-mos* mRNA after it has been amputated by injection of the anti-126 oligonucleotide, -126A. mRNA 1 contains 1,680 nucleotides of the luciferase gene followed by the last 321 nucleotides of the wild-type *c-mos* 3'UTR, including its polyadenylation signals (filled box). Polyadenylation signals include both AAUAAA and a CPE. mRNA 2 is identical, except that it lacks the last 83 nucleotides of the 3'UTR, which contain the polyadenylation signals. The prosthetic RNA is the same as in Fig. 5. *b*, Prosthetic RNA stimulate translation during maturation. Manually defolliculated oocytes were injected with 5 ng of either mRNA 1, mRNA 2 or mRNA 2 mixed with a threefold molar excess of prosthetic RNA. Half of the injected cells were incubated in progesterone ($104 \mu\text{g ml}^{-1}$) and the other half were not. At the end of maturation (twice GVBD₅₀), luciferase activity was measured as described¹². The experiment was repeated four times with oocytes from different frogs, using 15 to 25 oocytes per point. The magnitude of stimulation of mRNA 2 by the prosthetic ranged from three- to sixfold. A representative experiment is shown. The height of the vertical bars indicates the ratio of luciferase activity present in matured (+P) versus non-matured (–P) for each injected sample.



gene expression *in vivo*. *In vitro*, previous work demonstrated that two RNAs, by base-pairing, can form competent substrates for splicing³⁵ and nuclear polyadenylation³⁶. Unlike most previously described antisense RNA strategies used *in vivo*, the prosthetic RNA approach is not limited to inhibition of normal mRNA function. In principle, new activities may be imposed on natural mRNAs *in vivo*, in animal cells. As with all experiments that require formation of RNA–RNA duplexes *in vivo*, enzymes that destabilize or degrade RNA duplexes present potential complications. For example, many cells, including frog oocytes, contain an activity (dsRAD) that converts adenosines in double-stranded RNA to inosines, destabilizing the duplexes^{25,26}. This activity is nuclear in frog oocytes (as in most cells examined)²⁷, and so presumably does not interfere with the cytoplasmic RNA duplexes formed in our studies until after nuclear breakdown, by

which time *c-mos* translation has presumably already been activated.

In our experiments, the prosthetic RNA stimulates translation *in trans* by virtue of its polyadenylation signals. In the same way, other signals might be supplied that would control, for example, the location of the mRNA within the cell, or its stability. Signals that are normally present in the 3'UTR, which have now been discovered in a wide range of mRNAs and animal species^{9,28,29,34}, are ideal candidates to be supplied *in trans*. *A priori*, the target mRNA need only have its 3'UTR available for base pairing to the prosthesis.

Note added in proof: Since submission of this Article, cytoplasmic polyadenylation has been shown to be required for the control of *bicoid* mRNA translation and pattern formation in *Drosophila*³⁰ and of *c-mos* mRNA translation and meiotic maturation in mice³¹. □

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Crystal structure of an integral membrane light-harvesting complex from photosynthetic bacteria

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The crystal structure of the light-harvesting antenna complex (LH2) from *Rhodospseudomonas acidophila* strain 10050 shows that the active assembly consists of two concentric cylinders of helical protein subunits which enclose the pigment molecules. Eighteen bacteriochlorophyll *a* molecules sandwiched between the helices form a continuous overlapping ring, and a further nine are positioned between the outer helices with the bacteriochlorin rings perpendicular to the transmembrane helix axis. There is an elegant intertwining of the bacteriochlorophyll phytol chains with carotenoid, which spans the complex.

THE initial event in bacterial photosynthesis is the absorption of a photon by the light-harvesting antenna system, which is followed by a rapid and efficient transfer of this energy to the reaction centre, where 'trapping' occurs¹. Typically, purple bacteria contain two types of antenna complexes, both of which are integral membrane proteins². The first type, LH1, is intimately associated with the reaction centre forming the so-called 'core' complex. Arranged more peripherally to this, and present in variable amounts, is the second type, LH2. Both types of complex are built on a similar modular principle³. The light-absorbing pigments, bacteriochlorophyll *a* (Bchl *a*) and carotenoids, are non-covalently bound to two low-molecular-weight hydrophobic apoproteins, α and β . The native complexes are oligomers of these components.

Here we present the crystal structure of the LH2 (B800–850) complex from the purple non-sulphur photosynthetic bacterium *Rhodospseudomonas acidophila* strain 10050. The complex contains bacteriochlorophyll (Bchl) *a* and rhodopin-glucoside pigments⁴. The α -apoprotein contains 53 amino acids, and the β -apoprotein 41. Both proteins have been sequenced³.

The Bchl *a* molecules in this complex are divided into two spectral forms² which absorb at 800 nm and 850 nm. Circular dichroism analysis of these absorption bands has led to the conclusion that the Bchl *a* molecules that absorb at 800 nm are largely monomeric, whereas those absorbing at 850 nm are strongly exciton-coupled⁵. Conserved histidine residues in the apoproteins have been shown, by resonance Raman spectroscopy, to be liganded to the Mg at the centre of the Bchl *a* that absorbs at 850 nm⁶. The observation of efficient singlet-singlet energy transfer (>50%)⁷ from the carotenoid to Bchl *a* has been interpreted to indicate that the π -electron system in the two pigment types must be within van der Waals contact⁸.

Structure determination

Crystals of the LH2 complex from *Rps acidophila* strain 10050 were grown using a protocol based on that reported previously⁹. A marked improvement in the reproducibility and resolution of diffraction of the crystals was achieved using methods of separation based on size rather than charge. The integrity of the complex was monitored at each stage of the preparation by recording

the absorption spectrum over the range 250–900 nm. The characteristic Bchl *a* spectrum is sensitive to the precise environment of the Bchl *a*. Spectra recorded from crystals are identical to those from whole cells (unpublished data), indicating that both contain the same complex assembly. The complex forms tabular crystals, space group *R*32, with hexagonal cell dimensions $a = b = 120.3$ Å, $c = 296.2$ Å. The structure has been determined to a resolution of 2.5 Å with diffraction data collected at a synchrotron source, using multiple isomorphous replacement (MIR) methods. Table 1 gives the details and statistics for the data collection and phasing procedure.

The crystal asymmetric unit contains three protomer complexes, each of which consists of an α and a β apoprotein, three Bchl *a* molecules, one carotenoid and a detergent molecule (β -octylglucoside). The electron density map allows a tracing of residues 1–47 of the α -apoprotein, all of the β -apoprotein and all the pigments. Twenty-two water molecules have been positioned. Figure 1 shows two regions of the initial electron density map.

The overall assembly

The structure of the active assembly may be described simply. The transmembrane helices of nine α -apoproteins are packed side by side to form a hollow cylinder of radius 18 Å. The nine helical β -apoproteins are arranged radially with the α -apoproteins to form an outer cylinder of radius 34 Å. The α -apoprotein helices are parallel to the ninefold axis to within 2°, and the β -apoprotein helices are inclined by 15° relative to this axis. The pigment molecules are contained between these inner and outer protein walls. The Bchl *a*-binding histidines of the α (His 31) and β (His 30) apoproteins face outwards and inwards, respectively, forming a complete ring of 18 overlapping Bchl *a* molecules. For these molecules, the planes of the bacteriochlorins are parallel to the membrane normal and their centres are approximately 10 Å from the presumed periplasmic membrane surface. The nine remaining Bchl *a* molecules are packed between the β -apoprotein helices a further 16.5 Å into the membrane with their bacteriochlorin rings parallel to the membrane surface. There is an elegant intertwining of the carotenoid molecules and the phytol chains of both sets of Bchl *a* molecules. Figure 2 shows the arrangement of the nonameric assembly. The current agreement of 0.25 Å between the α -carbons of the three crystallographically independent protomers suggests exact ninefold symmetry of the assembly.

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TABLE 1 Crystallographic structure determination

Data set	No. crystals	Resolution (Å)	Completeness (%)	R_{merge} (%)	R_{iso} (%)	No. sites	Phasing power acentric (centric)	R_{cull}
Native(1)	1	3.00	99.0	3.0				
Native(2)	9	2.50	98.9	5.0				
K ₂ HgI ₄	1	3.00	93.4	3.6	11.7	6	0.9 (0.8)	0.83
Pt combined	1	3.00	90.6	3.7	12.8	6	1.4 (1.0)	0.71
K ₂ Pt(CN) ₄	1	3.00	96.8	3.3	13.0	3	1.0 (1.0)	0.67
K ₂ PtCl ₄	1	3.00	95.8	3.8	11.1	3	1.5 (1.2)	0.57

Data collection. Derivative soaks were characterized with data collected using a Siemens area detector on a rotating-anode source and an image plate on the Daresbury synchrotron radiation source (SRS). A native data set, and derivative sets for the K₂HgI₄ and Pt combined soaks were collected at station 9.6 at the Daresbury Synchrotron. Additionally, derivatized crystals were back-soaked in artificial mother liquor for short periods and data sets collected. All data were collected at a wavelength of 0.87 Å, providing anomalous signals close to optimal magnitudes for the derivatives. **Derivative data and Patterson solution.** Diffraction data from the 30 cm MAR image plate at station 9.6 were processed with the program MOSFLM¹⁴. Despite a high degree of thermal diffuse scatter, data of high quality were collected from single crystals including a native data set to 3.0 Å resolution. Difference Pattersons derived from structure factors of soaked and back-soaked data sets clearly showed a subset of heavy atom sites. The coordinates of these sites were obtained from SHELX-S¹⁵ in Patterson search mode. Cross-phasing using these subsets of sites with native and derivative amplitudes yielded all sites for the two derivatives. Subsequently the Pt combined soak was separated into the active reagents detailed above, and data sets were again collected under equivalent conditions at station 9.6. MIR sites were refined for the K₂Pt(CN)₄, K₂PtCl₄ and K₂HgI₄ derivatives to their isomorphous limit of 4.0 Å. **Phase improvement.** The MIR phases were extended to the derivative data limit and the phase set improved using solvent flattening and histogram matching^{16,17} phase extension. Solvent flattening procedures were undertaken with the program DM¹⁸. The mask was derived using the Wang¹⁹ method with a sphere of radius 10 Å and an estimated 55% solvent content (the estimate²⁰ from the calculated V_m is 73%). The resulting map showed all of the salient details of the complex and revealed a nonameric arrangement of protein subunits sharing a common rotation axis with the crystallographic three-fold. Non-crystallographic symmetry averaging was used to improve the map and extend the phase set to the limit of the native(2) data. The toroidal protein envelope of cylindrical radii 10 Å and 45 Å was subdivided into 40° segments and averaged with DM. The volume averaged comprised 3 × 13% of the unit cell. This resulted in an improved map with an R_{free} (ref. 21) of 23%, correlation coefficients in the range 0.95–0.96 between the three equivalent segments, and a mean figure of merit of 0.78 for the phase set. **Model building and refinement.** The map was of a very high quality with continuous density throughout. The crystallographic asymmetric unit of the complex was built with the program O²² using a combination of the solvent-flattened MIR map and the non-crystallographic symmetry-averaged map. The alignment of the protein sequence was aided by the location of α Met 1, and the well-characterized Bchl a -contacting histidines at α 31 and β 30. A seleno-methionine modified complex had been crystallized, and anomalous difference amplitudes from these crystals, phased with the MIR phase set, indicated the Met sulphur site in the native complex. The initial model (R_{cryst} 43%) was refined using several refinement strategies with the XPLOR²³ and PROLSQ²⁴ programs. Individual atomic temperature factors were refined isotropically. The current model comprises a total of 3,345 non-hydrogen atoms in the crystallographic asymmetric unit. The current R_{cryst} and R_{free} (calculated with 4.6% of reflections) over the resolution interval 12.0–2.5 Å are 23.7 and 29.2%, respectively, for 27,855 independent reflections ($F > 2\sigma F$). The diffraction patterns display pronounced thermal diffuse scatter, suggesting anisotropic thermal motion which the isotropic refinement has not modelled. The r.m.s. deviations of the model from target geometries are 0.023 Å for bond lengths and 3.4° for bond angles. The atomic coordinates will be deposited in the Brookhaven Protein Data Bank when the refinement is completed. All computations used programs from the CCP4 Suite¹⁸, except where explicitly stated. $R_{\text{merge}} = \sum_h \sum_j |I(h) - \langle I(h) \rangle| / \sum_h \sum_j I(h)$, where $I(h)$ is the mean intensity for reflection h , after rejection of outliers. $R_{\text{iso}} = \sum_h ||F_{\text{PH}}| - |F_P|| / \sum_h |F_P|$, where $|F_{\text{PH}}|$ and $|F_P|$ are, respectively, the derivative and native structure factor amplitudes. Phasing power = $\sum_h |F_{\text{H calc}}| / \sum_h \epsilon$, where $|F_{\text{H calc}}|$ is the calculated structure factor amplitude for the heavy atom, and ϵ is the residual lack of closure (of the vector triangle $|F_P|$, $|F_{\text{PH}}|$ and $|F_{\text{H calc}}|$) for reflection h (calculated for acentric and centric reflections over the resolution interval 16.0–4.0 Å). $R_{\text{cull}} = \sum_h ||F_{\text{PH}} - F_P| - |F_{\text{H calc}}|| / \sum_h ||F_{\text{PH}}| - |F_P||$, where $|F_{\text{PH}}|$, $|F_P|$ and $|F_{\text{H calc}}|$ are defined above (summed over centric reflections). Figure of merit = $\langle \cos(\Delta\alpha_h) \rangle$, where $\Delta\alpha_h$ is the error in phase angle for reflection h : a figure of merit of 0.78 corresponds to a phase error of 38.7°.

The arrangement of this assembly differs markedly from that in the plant light-harvesting complex¹⁰, where the gross assembly is formed from a trimer of complexes. Each monomer is formed from a single polypeptide chain forming a pair of tilted helices surrounded by pigment molecules and an adjacent third helix which is almost parallel to the membrane normal. The seven Chl a and five Chl b molecules in that structure have the planes of the porphyrins tilted to the membrane normal and are arranged in six separate clusters. Two carotenoid molecules span the membrane, contacting each Chl a and binding the core of helices together like a crossbrace.

The apoprotein subunits

Each apoprotein subunit forms a single helix spanning the membrane bilayer with the amino (N) terminus on the cytoplasmic side³. In the α -apoprotein the N-terminal residue is buried approximately 9 Å from the presumed membrane surface, and coordinates to the central magnesium atom of a Bchl a . Although the published amino-acid sequence³ has this N-terminal residue as methionine, the electron density is best fitted with a formylmethionine (fMet), where the Mg coordination is through the formyl oxygen (Fig. 1). The methionine sulphur atom is excluded as a possible ligand as its position has been accurately determined by a seleno-methionine derivative. The position of the sulphur atom restricts the possible movement of the terminal amine towards the Mg atom at the centre of the bacteriochlorin. A coordination, mediated by a water molecule, to the N terminus of a methionine residue was considered but

rejected as it does not give a satisfactory fit to this density. Residues 2 to 9 form nearly 3 turns of a 3_{10} -like helix. This amphipathic helix lies on the presumed membrane surface with Ile 6, Trp 7 and Val 9 directed into the hydrophobic interior, and the side chains of Gln 3, Lys 5 and Thr 8 extending below the presumed surface. There is a turn at Asn 11–Pro 12, allowing residues 11 to 36 to form the membrane-spanning helix. Another turn at Thr 38 leads to a second amphipathic helix lying along the presumed periplasmic surface of the membrane. Like the N-terminal helix, this has hydrophobic residues (Trp 40, Phe 41, Tyr 44, Trp 45) directed into the membrane and hydrophilic residues (Thr 39, Gln 46) above the presumed surface. These N- and carboxy (C)-terminal helices appear to anchor the trans-membrane helix nearly perpendicular to the membrane surfaces. The fold of the β -apoprotein is similar, although the shorter sequence is reflected in shorter N- and C-terminal locating structures. The first five residues of the β -apoprotein lie on the presumed membrane surface in an extended conformation with alternating hydrophobic residues (Ala 1, Leu 3, Ala 5) inserted into the membrane. Two turns of an irregular helix lead to the membrane-spanning α -helix from residue 11 to residue 36. A turn at Thr 37–Pro 38 leads to a short C-terminal tail where Pro 38, Trp 39 and Leu 40 anchor the helix to the presumed periplasmic membrane surface. Figure 3 shows the arrangement of the α - and β -apoproteins in the protomer.

Unexpectedly, the only direct interaction between the helices is at the N- and C-terminal ends. Within membrane-buried portions of the helices, interactions are mediated via the pigment

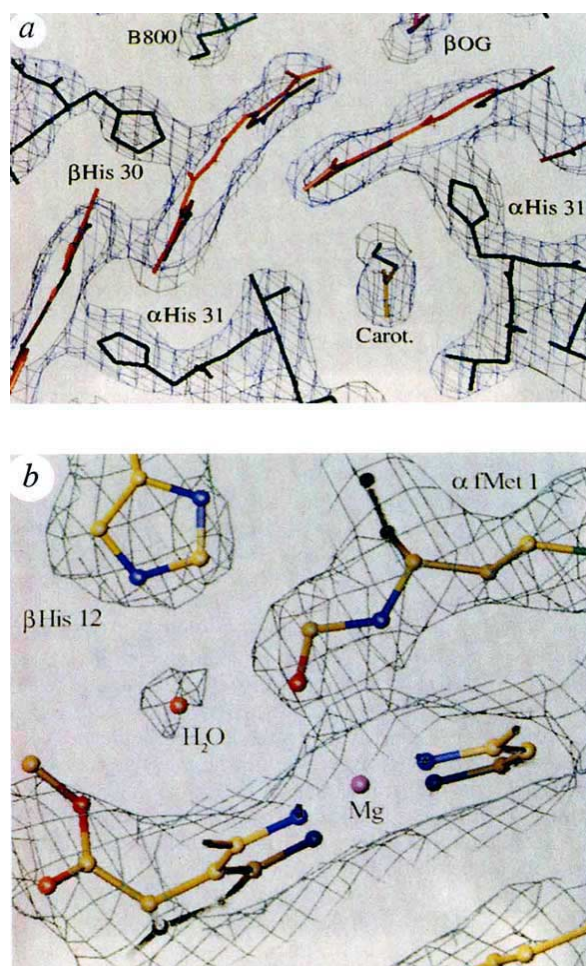


FIG. 1 Two regions of the electron density (MIR-phased, solvent-flattened, and averaged) used to determine the structure. *a*, The arrangement of apoproteins and B850 Bchl *a* molecules viewed down the crystallographic three-fold axis. Proteins are shown as black, B850 Bchl *a* red, carotenoids yellow, and the end of a B800 Bchl *a* phytol chain green. Adjacent to this a detergent molecule is coloured magenta. *b*, A B800 Bchl *a*, with the additional density at the N terminus of an α -apoprotein, is in the centre. A fitted formyl group and an adjacent buried water molecule are also shown. Atoms are colour-coded red for oxygen, blue for nitrogen, yellow for carbon, magenta for magnesium and green for sulphur.

molecules or buried water. This lack of helix-helix interaction, and the predominance of pigment-protein interactions, probably explains why all models¹¹ of the structure of this complex, which assumed helix-helix contacts, were wrong. At the N terminus, the association of the postulated α fMet 1 with a buried Bchl *a* restricts the protein-protein interactions to the radially associated α - and β -apoproteins. At the C terminus there are interactions between radial and adjacent α - and β -apoproteins and between adjacent α -apoproteins. In particular, the large aromatic residues (α Trp 40, Trp 45, Tyr 44, and β Trp 39) are involved in binding the apoproteins together through hydrogen bonds and hydrophobic interactions.

Apart from β Arg 20 and Asp 17, which form an ion pair on the outer face, both transmembrane surfaces of the nonamer are hydrophobic. No ordered molecules have yet been identified in the hydrophobic central channel of the complex.

Bacteriochlorophyll *a*

The Bchl *a* molecules form two groups. One group of nine well-separated molecules is located between the β -apoprotein helices, and another group of eighteen forms a closely interacting ring.

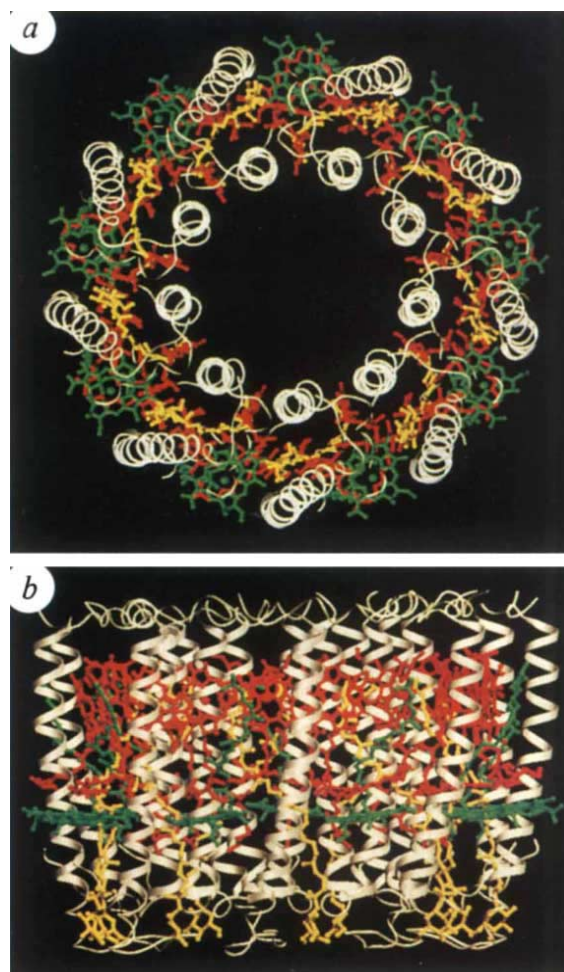


FIG. 2 *a*, The nonameric complex viewed from the cytoplasmic side of the membrane. Protein units are shown as white, B800 Bchl *a* green, B850 Bchl *a* red, and carotenoid yellow. *b*, The complex viewed perpendicular to the symmetry axis. Broad ribbons, beginning at the level of the amphipathic helix axis on the periplasmic side and at the level of a buried water molecule (not shown) on the cytoplasmic side, indicate the presumed hydrophobic region of the membrane.

Based on previous spectroscopic studies on this and several homologous LH2 complexes^{1-3,5,6}, the group of nine can be identified as the Bchl *a* molecules that absorb at 800 nm (B800 Bchl *a*) and the ring of eighteen as those that absorb at 850 nm (B850).

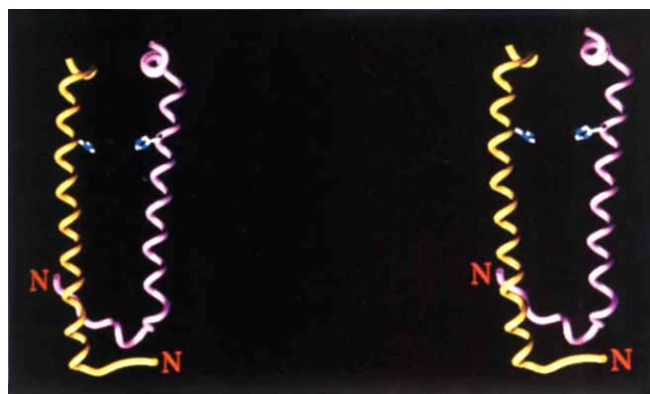


FIG. 3 A stereo view of the α (magenta) and β (yellow) apoproteins in the crystallographic protomer shown as a ribbon trace. The position of the His residues which ligand the B850 Bchl *a* molecules are shown.

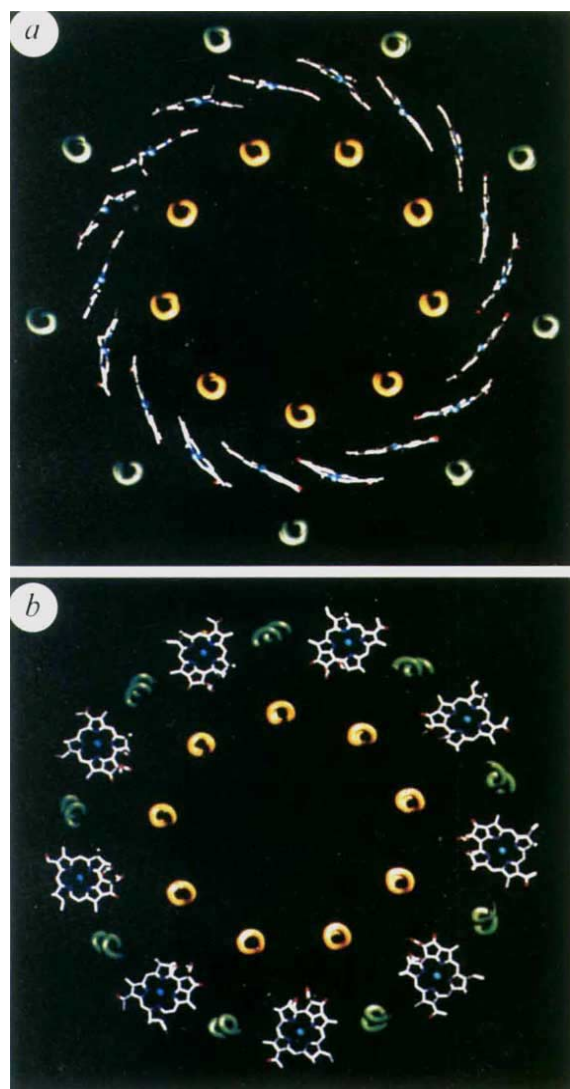


FIG. 4 *a*, The ring of B850 Bchl *a* molecules. *b*, The positions of the B800 Bchl *a* molecules between the helices of the β -apoproteins forming the outer cylindrical wall of the complex. The α -apoproteins are coloured yellow and the β -apoproteins green.

The eighteen B850 Bchl *a* molecules are supported by histidine residues on the α - and β -apoproteins with their bacteriochlorin rings perpendicular to the membrane surface and associated phytol chains descending into the hydrophobic core of the assembly. In contrast, the B800 Bchl *a* molecules have their bacteriochlorin rings aligned parallel to the membrane surface, are linked to a proposed formyl oxygen of an amino-terminal fMet of the α -subunit, and have their phytol chains directed up into the middle of the complex assembly.

Although the B850 nm Bchl *a* molecules form a complete overlapping ring (Fig. 4*a*), their molecular environments are not all equivalent. Within a protomer, the Mg–Mg distance is 8.7 Å, and between adjacent protomers, it is 9.7 Å. There are van der Waals contacts at the periphery of the bacteriochlorin systems. The conformations of the phytol chains of the α and β B850 Bchl *a* molecules differ (Fig. 5). In the α form the chain is slightly more extended, whereas in the β form the chain is bent to allow it to curve around the phytol chain rising from the B800 Bchl *a* coordinated to an adjacent α -apoprotein.

The nine B800 Bchl *a* molecules are located between β -apoproteins, with the planes of the bacteriochlorins parallel to the presumed cytoplasmic membrane surface and at a distance of about

11 Å from it (Fig. 4*b*). The distance between the central Mg atoms of these Bchl *a* molecules is 21 Å. The pigment is held in this position by coordination of the central Mg to the carbonyl oxygen of the putative N-terminal fMet as discussed above. Coordination from the N terminus to the central metal of a porphyrin/bacteriochlorin system is unusual and has been observed in only one other structure¹². The phytol chain projects up into the interior of the complex where it contacts the B850 Bchl *a* coordinated to the same apoprotein. The conformation of the chain differs again from that in the α and β B850 Bchl *a* molecules. Although the B850 molecules interact by overlapping at the edges of the bacteriochlorin systems, the closest interaction between B800 and B850 molecules is through the intertwined phytol chains. The phytol chain from the β B850 bacteriochlorophyll bends and passes parallel to the B800 bacteriochlorin ring at a distance of 4.0 Å, close enough for van der Waals contact. The distance between the central Mg atoms of the closest pair of B800 and B850 molecules is 17.6 Å.

There is a distinct difference in the environment of the two sets of Bchl *a* molecules. The pigments that absorb at 850 nm are in a hydrophobic environment, with α Ala 27, Ile 34, Trp 45 and β Phe 22, Ala 26, Trp 39 making a number of contacts. The only hydrogen bonds formed are from α -apoproteins: to the bacteriochlorin ring-B acetyl carbonyl oxygen from NE1 of Trp 45 for α -coordinated Bchl *a* molecules, and from OH of Tyr 44 (on an adjacent α -apoprotein) for β -coordinated ones. In contrast, the molecules that absorb at 800 nm are in a relatively polar environment, with water located between β His 12, the ring-D ester carbonyl oxygen, and the putative formyl group coordinated to the central Mg (Fig. 1). A strong hydrogen bond is formed from the Arg 20 side chain of a β -apoprotein to the ring-B acetyl carbonyl oxygen. There are few van der Waals contacts with hydrophobic protein residues.

Carotenoid

In the B800–850 LH2 complex of *Rps acidophila*, the carotenoid, rhodopin–glucoside, absorbs visible light. Estimates of the stoichiometric amount of carotenoid in the complex suggested a carotenoid:Bchl ratio of 1:2 (refs 2, 4) that is, there are 13–14 molecules for this assembly. The crystal structure shows one carotenoid molecule for each protomer of α - and β -apoproteins. It is uncertain whether the observed detergent molecule has replaced a second carotenoid in the protomer. The observed carotenoid is not associated with any single apoprotein. It has an extended conformation and spans the depth of the membrane, beginning with its glucosyl ring associated with Lys 5 and Thr 8 of an α -subunit. On its path through the complex, it makes

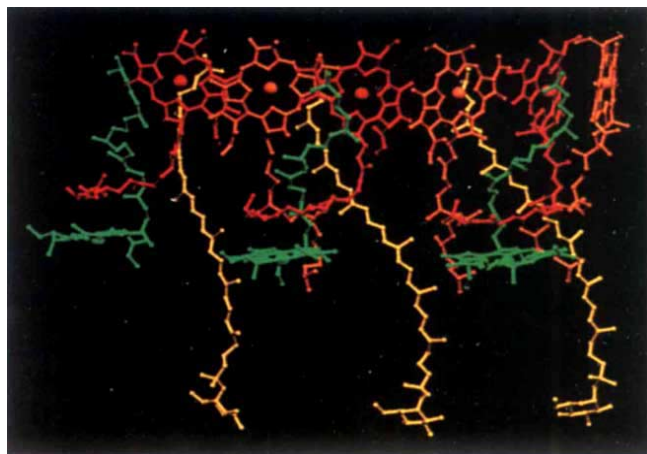


FIG. 5 The arrangement of the pigments in the asymmetric unit. B800 Bchl *a*, green; B850 Bchl *a* bound to α -apoproteins, orange; those bound to β -apoproteins, red; and carotenoid, yellow.

several van der Waals contacts ($<3.5 \text{ \AA}$) with hydrophobic residues of adjacent β and α apoproteins and with both B850 and B800 Bchl a molecules. It finishes with its terminal dimethyl groups in contact with His 31 of the adjacent α -apoprotein. It is this residue that is bound to the central Mg of a B850 Bchl a . The arrangement of the pigments in the asymmetric unit is shown in Fig. 5.

Energy transfer mechanism

When a Bchl molecule is excited by light, its first excited singlet state lasts for a few nanoseconds¹. The light-harvesting system must be able to transfer the absorbed energy to the reaction centre in a shorter time than this. Some of the important features that allow this to take place are revealed by the structure reported here.

Previous biophysical studies (reviewed in ref. 1) have shown that energy transfer within the LH2 complex can occur from the B800 to the B850 Bchl a molecules in 0.7 ps. Once the energy reaches the B850 molecules, it is rapidly transferred among them. This is seen as an ultrafast depolarization of the excited state on the 200–300 fs timescale. Energy transfer from LH2 to LH1 occurs in the 5–20 ps time range, but with LH2 alone the decay of the 850 nm excited singlet state takes 1.1 ns.

The ring of B850 Bchl a molecules acts rather like a 'storage ring', with the excited state rapidly delocalized over a large area.

The delocalization is facilitated by a highly hydrophobic environment which reduces the dielectric constant, allowing coupling over large distances. The energy is then available for transfer from any part of the ring to any neighbouring LH1 complex. It is clear from electron microscopy imaging of the LH1 complex¹³, and from a comparison of the primary structures³ of the LH2 and LH1 complexes, that the structure of the LH1 complex is similar to that of LH2 (with a larger ring). With such a ring structure, there is no requirement for the LH1 complex to have a special orientation to receive energy from the LH2 ring. Furthermore, because the B875 Bchl a molecules in LH1 are liganded to homologous histidine residues, as in LH2, it is likely that the B850 and B875 bacteriochlorophyll rings will be at the same point in the membrane. The overall effect will be to allow energy transfer from any LH2 to any LH1 complex that is within range, without regard to the orientation of either complex. This reduction in the dimensionality of the process will lead to a further kinetic gain.

Previous studies have shown that the carotenoid in this LH2 complex acts as an efficient accessory light-harvesting pigment ($>50\%$)⁷. The excited singlet lifetime of carotenoids is usually less than 10 ps⁸. Therefore, if energy transfer is to compete successfully with these rapid de-excitation processes, the carotenoid must be located very close to the acceptor bacteriochlorophylls⁸, as seen in the structure. $\square$

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LETTERS TO NATURE

Observational confirmation of a circumsolar dust ring by the COBE satellite

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ASTEROID collisions are an important source of the dust particles in the zodiacal cloud^{1–3}. These particles spiral in towards the Sun under the influence of drag forces^{4–6} and, in passing through the inner Solar System, are subject to gravitational perturbations by the planets, which may trap them (at least temporarily) in orbital

resonances^{7–10}. Recently, numerical simulations have shown that resonances with the Earth are particularly effective at trapping asteroidal dust, leading to the suggestion that the Earth may be embedded in a circumsolar ring of dust¹¹. The azimuthal structure of this ring was predicted to be asymmetric, with the region trailing the Earth being substantially more dense than that in the leading direction¹¹. This prediction is in both qualitative and quantitative agreement with the asymmetry in zodiacal light observed by the Infrared Astronomical Satellite (IRAS)^{11,12}, but the IRAS data alone are equivocal because of calibration uncertainties and sparse coverage of elongation angle¹². Here we report observations by the Diffuse Infrared Background Experiment¹³ (DIRBE) on the Cosmic Background Explorer satellite (COBE)¹⁴, which confirm both the existence of this ring and the predictions of its near-Earth structure.

DIRBE mapped the entire sky with a 0.7° beam in ten wavebands (1.25, 2.2, 3.5, 4.9, 12, 25, 60, 100, 140 and 240 μm). The DIRBE instrument measured the sky brightness relative to an internal zero-brightness reference source, thus eliminating the calibration uncertainty of IRAS. The viewing geometry for DIRBE is illustrated in Fig. 1. The COBE satellite orbits the Earth along the terminator, and it spins at 0.8 r.p.m. about an axis nearly perpendicular to the Earth–Sun line. The DIRBE field of view is offset 30° from the spin axis. The combination

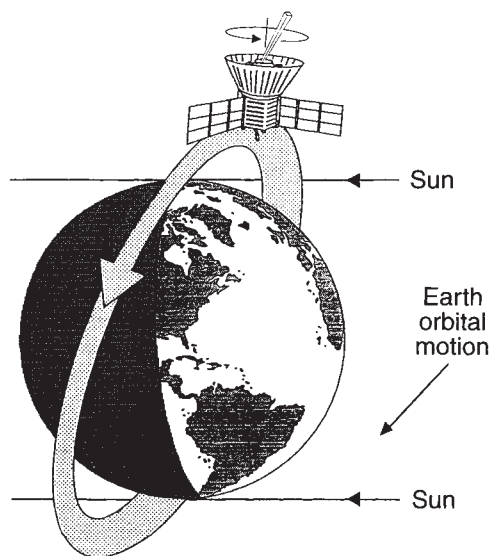


FIG. 1 The viewing geometry for DIRBE. The COBE satellite orbit is close to the terminator, and the spin axis is kept nearly perpendicular to the Sun. COBE is shown at one point in its orbit, with the DIRBE field of view extending outwards at an angle of 30° from the spin axis.

of orbital motion and spacecraft rotation causes the DIRBE field of view to trace a helical path on the sky within two viewing swaths, each 60° wide. These swaths, which correspond to observations with the satellite closer to the apex and antapex of the Earth's orbit about the Sun, are called the 'leading' and 'trailing'

swaths. Using one week of data, the two viewing swaths are well sampled. An example of a weekly map in the $12\text{-}\mu\text{m}$ waveband is shown in Fig. 2a. The position of the Sun has been rotated to the centre of the map for convenience, and lines of sight trailing the Earth, which have ecliptic longitudes greater than that of the Sun, appear left of centre.

First, we attempted to confirm the brightness asymmetry observed by IRAS, such that the zodiacal light was brighter when viewed along lines of sight trailing the Earth than when viewed leading the Earth. The peak zodiacal light brightness occurs at an ecliptic latitude that varies with time of year, and there is mid-infrared emission from Galactic dust, so we took care to separate these effects. We extracted DIRBE data in scans from the North Ecliptic Pole to the South Ecliptic Pole and back to the North again, in weekly intervals throughout the mission. Each scan was fitted with an empirical function that accurately matches the angular variation of the zodiacal light¹³. Portions of the scans within 20° of the Galactic plane were excluded, and a cosecant model of the emission from the Galaxy was fitted to the scans simultaneously with the empirical zodiacal light function. The polar brightnesses of the leading and trailing portions of the scan were forced to agree, as indeed they must. This removes one of the difficulties—namely scan-to-scan calibration errors—that was faced in measuring the trailing-leading asymmetry from the IRAS data¹². The peak zodiacal light brightness is shown as a function of time in Fig. 3. The brightness trailing the Earth was in fact greater than that leading the Earth throughout the mission by $\sim 2\text{--}3\%$, confirming observations by IRAS^{11,12}. The fact that the trailing scans are always brighter indicates that the brightness asymmetry is corotating with the Earth. A fixed longitudinal variation in the dust density would

FIG. 2 a, Map of the sky brightness observed by DIRBE in the $12\text{-}\mu\text{m}$ waveband during the one-week period beginning on 2 July 1990. The map is projected into ecliptic coordinates with the Sun in the centre and the North and South ecliptic poles at the top and bottom, respectively. The black region in the centre is the Sun-avoidance region. The two viewing swaths to the left and right of the Sun-avoidance region cover lines of sight trailing and leading the Earth, respectively. b, As a, but after the subtraction of a three-dimensional model for the interplanetary dust cloud. Note that the bulk of the sky brightness was subtracted, and the colour table was adjusted to reveal the much fainter residual brightness. The bright S-shaped ridge of emission from lower left to upper right is the Milky Way. The regions of enhanced brightness 90° from the Sun are the signature of the circumsolar dust ring.

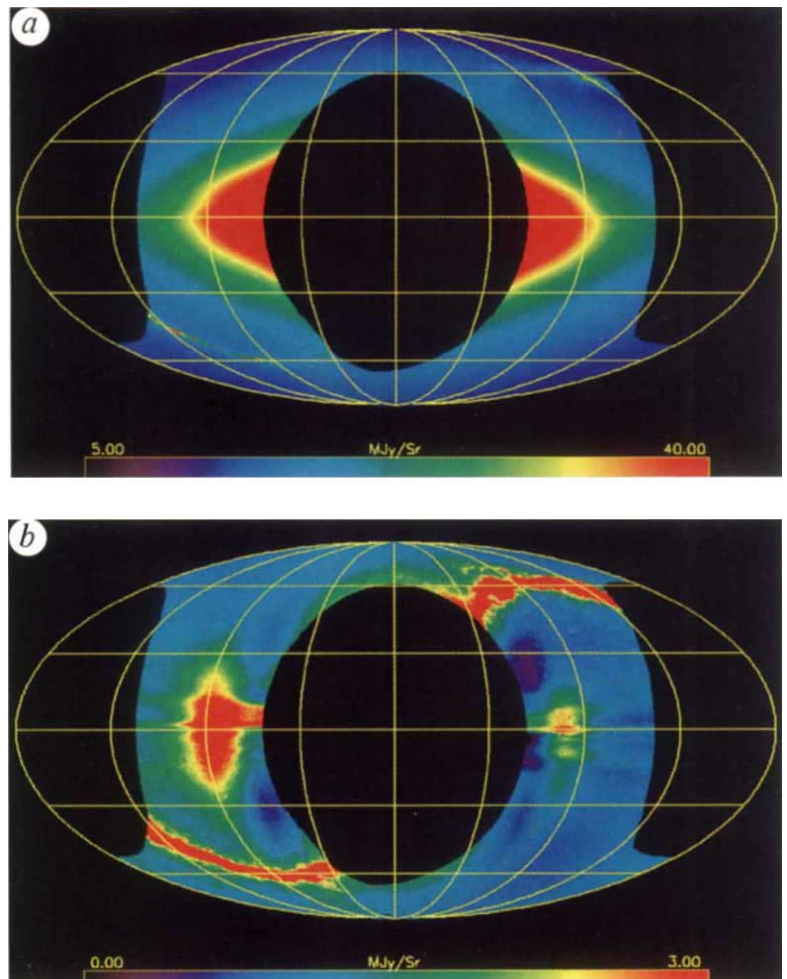
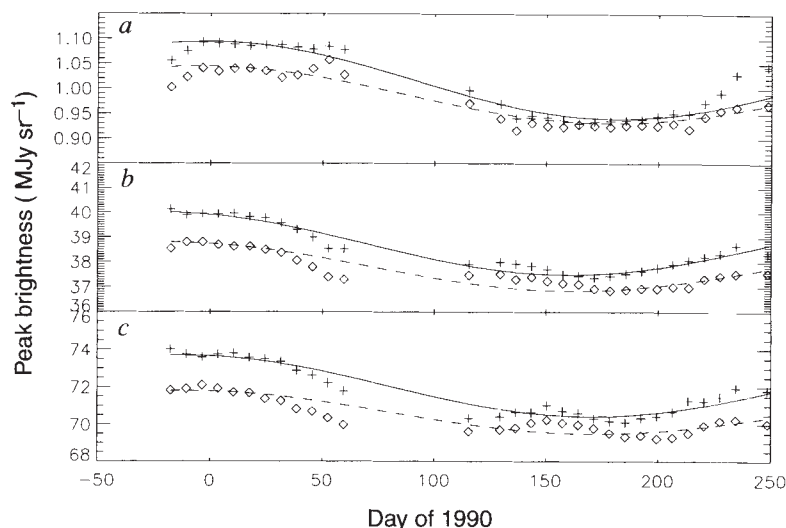


FIG. 3 The peak brightness of the zodiacal light observed at a solar elongation of 90° is shown as a function of time throughout the DIRBE mission at three wavelengths: a, $4.9\ \mu\text{m}$; b, $12\ \mu\text{m}$; c, $25\ \mu\text{m}$. Gaps in the plotted data are times when the Galaxy could not be easily separated from the zodiacal light. The brightness trailing the Earth (+symbols) was brighter than that leading the Earth ($\diamond$) throughout the mission, demonstrating that the region of enhanced brightness followed the Earth. The curves drawn through the data are sinusoidal fits, with a period of one year, for the trailing (solid curve) and leading (dashed) brightness.



cause the trailing scans to be brighter for six months, then the leading scans brighter for the other six months. The trailing-leading brightness difference is apparently not constant, being largest when the Earth is near perihelion and smallest when the Earth is near aphelion. The temporal variation of the trailing-leading brightness difference may be due to the motion of the Earth with respect to the dust ring—an effect that is predicted based on the numerical simulations¹¹.

Using the trailing-leading brightness asymmetry, we measured the temperature and albedo of the grains in the dust ring. In January 1990, the trailing-leading brightness differences were 0.05 ± 0.01 , 1.1 ± 0.2 and $1.7 \pm 0.1\ \text{MJy sr}^{-1}$ ($10^{-17}\ \text{erg cm}^{-2}\ \text{s}^{-1}\ \text{Hz}^{-1}\ \text{sr}^{-1}$) at wavelengths of 4.9, 12 and $25\ \mu\text{m}$, respectively. The colour temperature of the brightness asymmetry is $290 \pm 15\ \text{K}$, similar to the temperature of the zodiacal light¹⁶ and comparable to expectations for particles, $10\text{--}100\ \mu\text{m}$ in radius, composed of at least moderately absorbing material¹⁵. The trailing-leading asymmetry is marginally detected at $1.25\ \mu\text{m}$, with a brightness of $0.013 \pm 0.003\ \text{MJy sr}^{-1}$ indicating a geometric albedo of 0.3 ± 0.1 , consistent with that of the other cloud particles¹⁶.

Using the weekly sky maps created by DIRBE, we were able to image for the first time the structure responsible for the trailing-leading brightness asymmetry. To remove the emission from relatively smoothly distributed dust, we have fitted a model of the sky brightness based on integrating along each line of sight a parametrized model for the density and temperature of interplanetary dust. The cloud was assumed to be cylindrically symmetric as well as reflection-symmetric about a midplane, with a density of the form^{17–19}

$$n(r, z) = n_0 r_{xy}^{-\alpha} \exp(-\beta |z/r_{xy}|^\gamma) \quad (1)$$

where r_{xy} is the distance from the cloud centre in the midplane, and z is the vertical distance from the midplane. The location

of the cloud centre with respect to the Sun and the tilt and ascending node of the midplane were fitted. The grain temperatures were assumed to vary as $T = T_0 r^{-\delta}$, with $T_0 = 286\ \text{K}$ determined from the $4.9\text{--}25\ \mu\text{m}$ colour. Scattering in the near-infrared (1.2 , 2.2 and $3.5\ \mu\text{m}$) was modelled using a scattering phase function consisting of three Henyey–Greenstein functions²⁰. The albedo in each near-infrared band and the emissivity in each of the thermal emission bands ($3.5\text{--}240\ \mu\text{m}$) were free parameters. In addition to the smooth cloud model, the band-pair that appears near $\pm 10^\circ$ was fitted using a parametrized form based on the ‘migrating’ band model⁶.

The smooth cloud model was fitted to lines of sight observed leading the Earth only, because the trailing lines of sight are predicted to be more affected by the dust ring. The parameters $\alpha = 1.39 \pm 0.03$, $\beta = 3.26 \pm 0.04$, $\gamma = 1.02 \pm 0.01$ and $\delta = 0.42 \pm 0.01$ were optimized, and the density at $1\ \text{AU}$ is $n_0 \sigma = (7.6 \pm 0.1) \times 10^{-21}\ \text{cm}^{-1}$ —some 65% larger than that inferred from *in situ* observations of meteoroids²¹. The residual map, after the smooth cloud model was subtracted from the map in Fig. 2a, is shown in Fig. 2b. This Figure should be compared with the simulated all-sky view given on the cover of the 30 June 1994 issue of *Nature*¹¹. Two regions of enhanced brightness leading and trailing the Earth are evident, with the latter region substantially brighter. The region of enhanced brightness trailing the Earth is centred $\sim 90^\circ$ from the Sun, and that leading the Earth is centred $\sim 80^\circ$ from the Sun. The full-width at half-maximum brightness of the trailing region is 30° in latitude and 15° in longitude. These observations are in remarkable agreement with the predictions for small particles in orbital resonance with the Earth¹¹. Based on the excess brightness of the trailing region and its predicted size along the line of sight, its peak volumetric cross-section (density times cross-section) is $\langle n \sigma \rangle \approx 3 \times 10^{-21}\ \text{cm}^{-1}$, nearly half that of the smooth cloud model at $1\ \text{AU}$. $\square$

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A unified picture of the crystal structures of metals

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THE crystal structures of the light actinides have intrigued physicists and chemists for several decades¹. Simple metals and transition metals have close-packed, high-symmetry structures, such as body-centred cubic, face-centred cubic and hexagonal close packing. In contrast, the structures of the light actinides are very loosely packed and of low symmetry—tetragonal, orthorhombic and monoclinic. To understand these differences, we have performed total-energy calculations, as a function of volume, for both high- and low-symmetry structures of a simple metal (aluminium), a non-magnetic transition metal (niobium), a ferromagnetic transition metal (iron) and a light actinide (uranium). We find that the crystal structure of all of these metals is determined by the balance between electrostatic (Madelung) interactions, which favour high symmetry, and a Peierls distortion of the crystal lattice, which favours low symmetry. We show that simple metals and transition metals can adopt low-symmetry structures on expansion of the lattice; and we predict that, conversely, the light actinides will undergo transitions to structures of higher symmetry on compression.

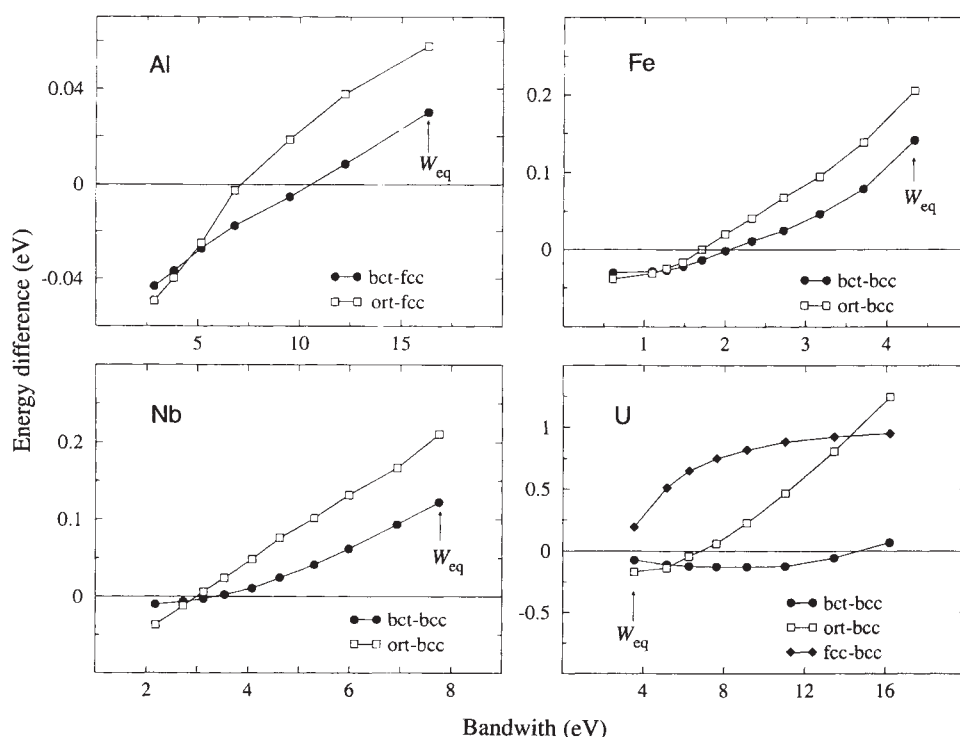
On solidification the metallic elements display a number of crystal structures, a wide range of bonding energies (cohesion), as well as a multiplicity of elastic properties. At room temperature, the simple metals condense in symmetrical, close-packed structures. Early theoretical work, which made use of pseudo-potential theory, successfully reproduced the structural stabilities

of these simple metals². The viewpoint that has emerged is that the electrons move more or less freely in the lattice; all ground-state properties can be understood from this picture, including the crystal structure. The bonding of the *d* transition metals is more complex and characterized by a rather broad *sp* band which is intersected by a narrow *d* band. It is the filling of first bonding and then antibonding orbitals of the *d* band in the transition metals that leads to the well known parabolic trend in cohesion, bulk modulus and lattice constant for these elements³. Next, it is a combination of *d*-band filling and the characteristic shape of the body-centred cubic (b.c.c.), face-centred cubic (f.c.c.) and hexagonal close packed (h.c.p.) density of states that leads to the understanding of the observed crystal-structure sequence⁴ (h.c.p.→b.c.c.→h.c.p.→f.c.c.) when the series are traversed.

We now turn to the *4f* and *5f* series of metals—respectively the lanthanides and actinides. The localized behaviour of the *4f* electrons, being part of a chemically inert core, leads the chemical bonding and crystal structures of the lanthanides to be similar to those of the transition metals^{5–7}; the magnetic properties originate from the localized *4f* states, but the bonding is determined by the *d* bands. The cohesive properties and crystal structures of the *4f* elements correspond directly to those of the transition metals which have the same number of *d* electrons. Similarly the *5f* electrons in the late actinides, from americium onwards, are localized and these metals show a close relationship to the lanthanides in this context^{7–9}. This leads again to *d*-bonding characteristics and the stabilization of close-packed crystal structures. The behaviour of the crystal structures of the *sp* metals, *d* transition metals, lanthanides and the heavier actinide metals is thus well understood and for these materials one encounters close-packed structures. In contrast to the heavy actinides, the *5f* electrons in the light actinides participate in bonding, in a manner similar to the dominant *d*-electron bonding in the transition metals. The remaining puzzle is therefore the distorted structures observed for the light actinide metals (Th–Pu), which have no counterpart among the rest of the metallic elements.

To make progress towards a unified picture of the crystal structures of the metallic elements, we note two important

FIG. 1 Calculated energy differences between the α -U structure (orthorhombic, ort) and the b.c.t. structure relative to the f.c.c. (Al) and b.c.c. structure (Fe, Nb and U) as a function of bandwidth of the *p* states (Al), *d* states (Fe and Nb) and *f* states (U). For U, the f.c.c.–b.c.c. energy difference is also shown. For all calculations the *c/a* ratio in the b.c.t. structure has been kept fixed at ~ 0.8 which corresponds to the value observed for b.c.t. Pa (a *5f* metal), whereas the *b/a* ratio, *c/a* ratio and positional parameter of the α -U structure were kept fixed to the values observed in U at low temperatures. W_{eq} denotes the bandwidth at the equilibrium volume and was evaluated from the Wigner–Seitz rule. The total-energy calculations were done using a full-potential linear ‘muffin-tin’ orbital method based on the local density approximation. The basis functions, charge density and potential were expanded in a Fourier series in the interstitial, and in a spherical harmonic series inside spheres centred at each atomic position. The calculations employed a double basis set, and were thus very well converged.



differences between the earlier actinides and the rest of the metals. Namely the disparity of the orbitals involved in the chemical bonding, that is, the angular behaviour of d versus the f valence states, as well as the narrower bandwidth of the $5f$ states. The latter is $\sim 1\text{--}3\text{ eV}$, compared to the d -bonded materials which have a d bandwidth of $\sim 3\text{--}10\text{ eV}$, and the sp metals where the bandwidth is even larger. (There is a change of viewpoint here; compared to the $5f$ states we now consider the d bands to be broad.) This observation motivates a study of the structural stabilities of these materials under conditions where their bandwidths are similar, that is, a volume expansion of the sp - and d -bonded metals and a compression of the $5f$ metals.

Because we want to study sp and d metals at expanded volumes, where the bandwidths decrease to values similar to the f bandwidth of the early actinides, we first note that any metal can become magnetic¹⁰ on expansion. However, the light actinides show (somewhat surprisingly) a weaker tendency to form a magnetic state than do, for instance, the $3d$ elements; this is so even though the $5f$ bandwidth is similar to (or even smaller than) the $3d$ bandwidth. The explanation for this is that the exchange integral of the f states (Th–Pu) is only about half of the exchange integral of the d states in, for instance, Ni¹¹. This dissimilarity, however, does not affect our arguments for the stabilization of close-packed, high-symmetry structures versus open-packed, low-symmetry structures; and we will demonstrate this below using a ferromagnetic $3d$ metal (Fe) as an example.

We choose systems that can be regarded as typical examples of their respective series. Aluminium (f.c.c.) represents an sp metal, Nb (b.c.c.) a paramagnetic d transition metal, Fe (b.c.c.) a ferromagnetic d transition metal, and U (α -U, orthorhombic) a $5f$ metal. The ground-state crystal structures of these metals are compared to the body-centred tetragonal (b.c.t.) and α -U structures which are typical structures for an f -bonded metal. This detailed comparison has been done by means of first-principles total-energy calculations using the linear 'muffin-tin' orbital method with a self-consistently calculated potential, which has a general shape (ref. 12 and J.M.W., unpublished results). The atomic number, the crystal structure, and the atomic volume are the only input data.

In Fig. 1 we show for Al, Fe, Nb and U the stability of the b.c.t. and α -U (orthorhombic) structures relative to the f.c.c. and the b.c.c. structure as a function of bandwidth. The behaviour of the different types of metals is very similar. For narrow bandwidths the open-packed, low-symmetry orthorhombic structure is stable, whereas intermediate bandwidths favour the more symmetrical and close-packed b.c.t. structure. At broad bandwidths, all the metals in Fig. 1 have transformed to one of the closer packed crystal structures with higher geometrical symmetry (b.c.c. or f.c.c.). The bandwidth at the equilibrium volume (W_{eq}) has also been marked in this figure. For Al, Fe and Nb the bandwidth at equilibrium is sufficiently broad to stabilize a high-symmetry structure, whereas for U the bandwidth is more narrow and this favours the open, low-symmetry structure. Thus the sp , d and f metals display a similar crystal-structure sequence as a function of bandwidth (or volume), and the angular dependence of the different valence electron orbitals is not the crucial parameter. The reason for the appearance of the open structures in the light actinides is simply that they happen to condense at a volume where the $5f$ bandwidth is sufficiently narrow. Our results for compressed uranium metal are therefore especially interesting, because the predicted crystal-structure sequence under compression (α -U $\rightarrow$ b.c.t. $\rightarrow$ b.c.c.) can be verified experimentally. The first transition in U, at $\sim 0.8\text{ Mbar}$, should be accessible with present high-pressure techniques and we hope that our prediction will stimulate experimental work.

Thus, when the bands of the electron states which dominate the chemical bonding (sp , d or f) are sufficiently narrow, one

predicts crystal structures of the same type for all metals (simple metals, transition metals and actinide metals). We now study the different energy contributions that determine the crystal structure. The general shape of the density of states for a given crystal structure is approximately the same at all volumes; the significant change between the low- and high-volume cases is mainly a modification of the bandwidth. The additional consideration necessary to understand the crystal-structural stability of narrow-band metals is the Peierls distortion¹³. It is well known that a one-dimensional lattice can lower its total energy by means of dimerization. The argument for the crystal-structure distortion in one-dimensional systems will here be used for three-dimensional lattices. The one-particle energy (E_b) contribution to the total energy is the sum of all the occupied valence energy levels. If the system attains a high-symmetry structure there will be degenerate energy levels. If these levels lie close to the Fermi level (E_F —the highest occupied level) the one-particle energy, E_b , can be lowered by means of a crystal-structure (Peierls) distortion. This is because in this process some energy levels will be pushed above E_F and thereby become unoccupied, whereas other levels will be pushed down in energy and therefore energy is gained. This mechanism is especially effective if there are many degenerate energy levels around E_F , that is, if the energy bands are narrow. For broader bands less energy is gained from a Peierls distortion, as there are fewer energy levels around E_F that can affect E_b by the described symmetry-breaking mechanism. This tendency of the one-electron term to favour low-symmetry structures is balanced by the electrostatic (Madelung) energy, which stabilizes high-symmetry structures⁴. Thus for metals with narrow bands lying close to E_F , as for the light actinides under ambient conditions, the one-electron term dominates and a gain in energy can be achieved by a Peierls distortion. On the other hand, for the sp and d metals at ambient conditions the bands are too broad for E_b to significantly lower the energy by this process, and instead the Madelung contribution dominates.

These results indicate that the factors that determine the various crystal structures are essentially the same for all elemental metals, and that the determining parameter is the width of those electron states that dominate the chemical bonding. High-pressure experiments on the light actinides would be most useful as they could clarify the details of this behaviour. Based on our findings for uranium, we predict that all delocalized f elements will be stabilized first in the b.c.t. (or similar) structure, and then in high-symmetry, close-packed structures, as a function of increasing pressure. Conceptually, such behaviour provides the link between the crystal structures of the light actinides, the d transition metals, and the simple metals. $\square$

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A covalent micro/nano-composite resistant to high-temperature oxidation

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ADVANCED ceramic materials that can withstand high temperatures (over 1,500 °C) without degradation or oxidation are needed for applications such as structural parts for motor engines, gas turbines, catalytic heat exchangers and combustion systems^{1,2}. Hard, oxidation-resistant ceramic composites and coatings are also in demand for use on aircraft and spacecraft. Silicon nitride (Si_3N_4) and silicon nitride/carbide ($\text{Si}_3\text{N}_4/\text{SiC}$) composites are good candidates for such high-temperature applications^{2,3}. Commercial Si_3N_4 parts can be used in oxidizing environments up to 1,200–1,300 °C (ref. 4), but are oxidized at still higher temperatures. Here we describe the synthesis of a covalent ceramic composite which is resistant to oxidation at temperatures up to 1,600 °C. The composite is formed from an amorphous silicon carbonitride, which crystallizes at high temperature into a composite of α - Si_3N_4 microcrystals and α -SiC nanocrystals. The oxidation resistance stems from the formation of a passivating surface layer of SiO_2 a few micrometres thick.

The starting material, dense bulk amorphous silicon carbonitride with the general formula $\text{Si}_{3+x}\text{N}_4\text{C}_{x+y}$, was synthesized according to the method described in ref. 5. This involved the pyrolysis of compacted polyhydridomethylsilazane powder (with the composition $[\text{CH}_3\text{SiH-NH}]_m[(\text{CH}_3)_2\text{Si-NH}]_n$; Nichimen Corp., Tokyo) at 1,000 °C under argon. The reaction product consisted of an amorphous and single-phase silicon carbonitride matrix with the molar composition $\text{Si}_{1.7}\text{C}_{1.0}\text{N}_{1.6}$ (refs 5, 6).

To study the high-temperature stability of the material, we prepared cylindrical samples 1 cm in height and 1 cm in diameter. The porosity was 7% and the mean pore radius and the specific surface area, analysed by mercury porosimetry, were 75 nm and $1 \text{ m}^2 \text{ g}^{-1}$, respectively. The samples were pre-treated in nitrogen at 1,400 °C for 50 h before oxidation. The oxidation behaviour was studied by thermogravimetric analysis in the temperature range 1,000–1,400 °C. The samples were heated to the final temperature in nitrogen at a rate of 30 K min^{-1} , isothermally annealed for 24 h under flowing air and finally cooled to room temperature. Investigations at higher temperatures (1,500–1,700 °C) were conducted in air, in an alumina furnace. In this case, the samples were heated at 5 K min^{-1} and isothermally annealed in air for 50–60 h at the final temperature. To avoid reaction between the sample and the alumina crucible, the silicon carbonitride was protected by a platinum foil.

Above 1,400 °C, the material begins to crystallize in an argon or nitrogen atmosphere^{5,6}, accompanied by significant loss of nitrogen from the sample (8 wt% (calc.: 10 wt%) at 1,850 °C under 0.1 MPa N_2). As a consequence, the porosity and the mean pore radius increase from 7% and 75 nm in the as-synthesized silicon carbonitride (1,000 °C, Ar) to 24% and 100 nm after heating the sample up to 1,850 °C. This is the result of *in situ* crystallization to Si_3N_4 and carbon formed during phase partitioning of silicon carbonitride according to equation (1). Simultaneously, C reacts with Si_3N_4 to give SiC

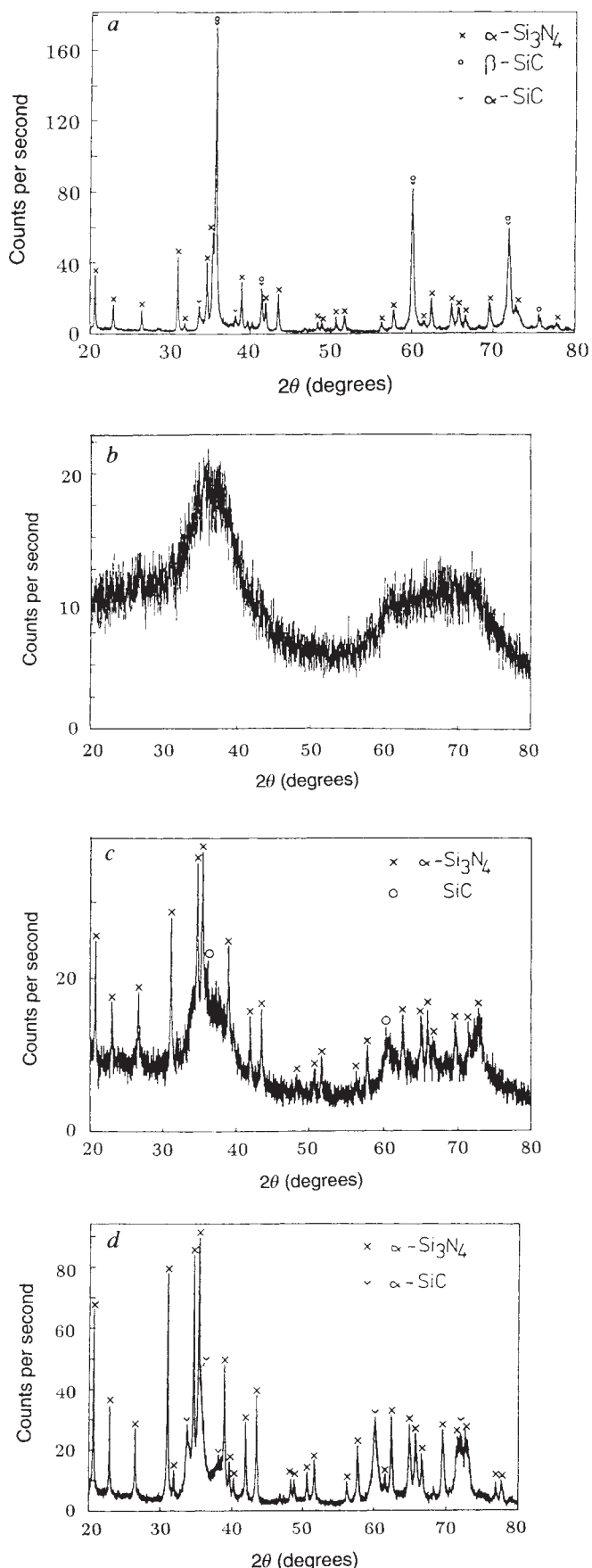


FIG. 1 X-ray diffraction spectra of silicon carbonitride heat-treated at 1,500 °C in nitrogen (a) and in air (b). Also shown are spectra of the same material heat-treated in air at 1,600 °C (c) and 1,700 °C (d). Isothermal holding time at temperature, 50 h.

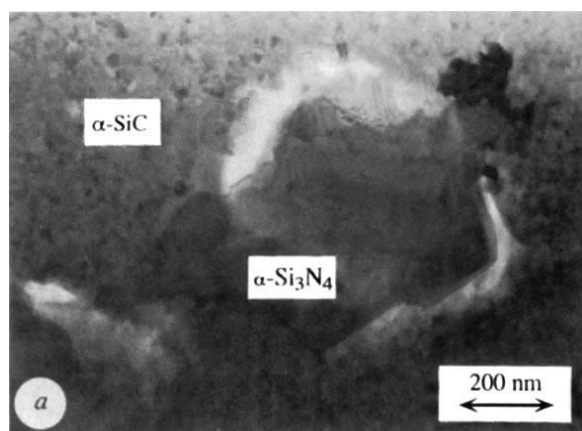
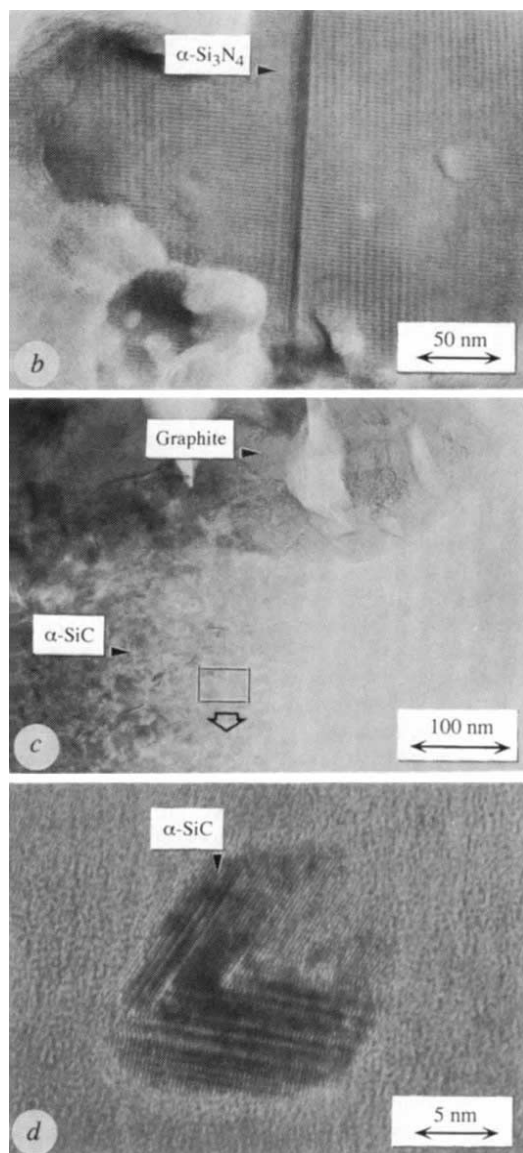
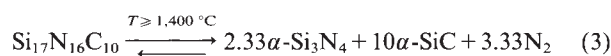
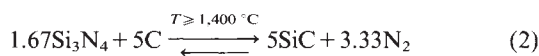
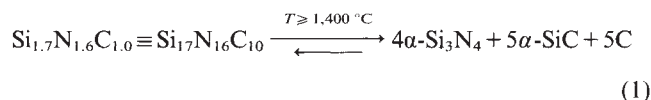


FIG. 2 TEM and HREM micrographs of the micro/nano-composite formed after oxidation of silicon carbonitride at 1,600 °C for 50 h in air. *a*, α - Si_3N_4 particle surrounded by fine-grained SiC; *b*, an α - Si_3N_4 microcrystal shown at higher magnification; *c*, Graphite precipitation close to α -SiC; *d*, HREM image of nanosized α -SiC (with numerous stacking faults); see also boxed area in *c*. It should be noted that phase identification of all crystalline phases was confirmed by selected-area electron diffraction (microdiffraction for nanosized α -SiC particles) in parallel with electron energy-loss spectroscopy. The occurrence of regions with amorphous-like contrast within the TEM images (compare also *d*) are due to crystallites which are not sufficiently close to a Bragg orientation. Based on the TEM observations, it is concluded that this material is $\geq 96\%$ crystalline.



and N_2 (equation (2)) which are the thermodynamically stable products⁷ at $T \geq 1,440$ °C.



The overall solid-state reaction given in equation (3) can be verified quantitatively by chemical analysis (Table 1).

We found that this undesired degradation of silicon carbonitride in an inert environment above 1,400 °C can be circumvented by heating the material in air. No significant weight change, and hence no decomposition, can be detected

up to 1,600 °C. According to X-ray powder diffraction and transmission electron microscopy (TEM) the polysilazane-derived silicon carbonitride, $\text{Si}_{1.7}\text{C}_{1.0}\text{N}_{1.6}$, annealed in air above 1,500 °C showed evidence of the formation of crystalline

TABLE 1 Analyses of heat-treated $\text{Si}_{3+x}\text{N}_4\text{C}_{x+y}$

Heat treatment			Composition (wt%)				
Temp. (°C)	Time (h)	Gas	Si	C	N	O	Total
1,000	1	Ar	57.8	14.3	26.0	0.8	98.9
1,400	50	N ₂	57.0	14.4	26.5	1.5	99.4
1,500	50	N ₂	65.0	17.4	16.6	0.6	99.6
1,600	50	Air	58.5	12.3	25.6	3.3	99.7

Chemical analyses of silicon carbonitride, heat-treated at various temperatures and in various atmospheres. Silicon was determined by optical emission spectrometry with inductively coupled plasma excitation (Perkin Elmer ICP 5500; standard deviation, <1% with Cu as internal standard); carbon, nitrogen and oxygen were analysed by carrier gas heat extraction (LECO C, S-analyser 244 and N, O-analyser TC 436; standard deviation, <1% for C and <2% for N and O).

FIG. 3 Cross-section of silicon carbonitride oxidized at 1,400 °C for 24 h, showing a dense 2- μ m-thick SiO₂ surface layer. The SiO₂ and Si-C-N regions identified were determined by analysis of the oxygen and nitrogen distribution by wavelength dispersive X-ray analysis. X-ray diffraction studies of the oxidized surface showed the presence of α -cristobalite as the crystalline phase, exclusively. This finding also corresponds with X-ray photoelectron spectroscopy measurements revealing pure SiO₂ at the surface.



phases— α -Si₃N₄, α -SiC and graphite (Figs 1 and 2). Chemical analysis reveals that the composition after 50 h oxidation at 1,600 °C resembles that of the starting material, demonstrating the high thermal compositional stability of dense bulk silicon carbonitride under oxidizing conditions (Table 1).

The relative mass gain after 60 h isothermal oxidation at 1,600 °C was found by analysis to be 0.4%, and was attributed to the formation of a dense SiO₂ layer (Fig. 3). The enhanced thermal stability of the composition of bulk silicon carbonitride containing a protective silica surface layer can be explained by the low nitrogen diffusion coefficient⁸ through SiO₂ which hinders the solid-state reaction (equation (2)). Thus the equilibrium of the reaction given in equation (2) is shifted to the side of the reactants, enabling the formation of novel metastable bulk composite materials in the ternary system Si-C-N with Si₃N₄, SiC and C as discrete phases (equation (1)). Moreover, because of the low oxygen diffusion coefficient⁸ of 10^{-11} – 10^{-6} cm² s⁻¹ at 900 and 1,400 °C, respectively, the passivating dense SiO₂ layer effectively protects the bulk silicon carbonitride from further oxidation.

According to thermal gravimetric analysis, the mass gain related to the initial mass amounted to 0.7% at 1,000 °C, 0.8% at 1,100 °C, and 0.13% at 1,400 °C. The higher mass gain found at lower oxidizing temperatures is associated with Knudsen diffusivity and with pronounced sealing of the pore mouth with increasing temperature resulting in a lower depth of oxidation in the pore channel. The porosity decreases from 7% (mean pore radius, 75 nm) measured for the unoxidized sample to 3.6% (mean pore radius, 5 nm) of the material oxidized at 1,300 °C in air for 24 h. This result is in good agreement with the investigations on the oxidation behaviour of reaction-bonded Si₃N₄ reported by Porz and Thümmel⁹. The analysis of the mass change could not be related to the surface area as the value of the latter is not constant during the measurement. The relative mass gain versus time at different constant temperatures exhibit an initial parabolic character. In the case of 1,200–1,400 °C, the initial parabolic curves change to asymptotic curves after 40 min (1,400 °C) and 120 min (1,200 °C), respectively. Thus the reaction mechanism changes with time from reaction-controlled to diffusion-controlled oxidation. The rate of mass increase then slows to nearly zero, indicating SiO₂ surface passivation. Further improvement of the exceptional high-temperature properties may be achieved by reducing the porosity of the as-synthesized silicon carbonitride (for example, by post hot-isostatic pressing

or by infiltration of the pore volume with polysilazane and subsequent pyrolysis of the polymer fraction).

Detailed TEM and scanning electron microscopy (SEM) investigations revealed the formation of a covalent micro/nano-composite after oxidation at 1,600 °C for 50 h. The assemblage consists of α -Si₃N₄ particles a few micrometres in size, α -SiC nanocrystals and residual graphite (200–500 nm) (Fig. 2).

The material investigated here underwent complete crystallization during oxidation at elevated temperatures without changes in phase composition. No residual amorphous Si_{3+x}N₄C_{x+y} matrix material was observed by TEM. Following the microstructural development of this material by investigating TEM foils prepared from samples heat-treated under various conditions, the evolution of the resulting micro/nano-structure is as follows. Heat-treatment at 1,400 °C (under nitrogen) results in a nearly completely amorphous Si_{3+x}N₄C_{x+y} matrix, as seen by the phase contrast in high-resolution electron microscopy as well as the corresponding electron diffraction pattern, which is consistent with the X-ray diffraction results. Small precipitates of α -Si₃N₄, α -SiC and graphite are already observed in the amorphous matrix at this stage, but the amount of crystalline phases is very small and below the level of detectability by X-ray diffraction. On further oxidizing annealing, the initially formed α -Si₃N₄ grows continuously, forming large α -Si₃N₄ microcrystals. The SiC nuclei, observed after heat treatment at low temperatures, may act as nucleation sites for the further crystallization of α -SiC; crystals of the latter are 10–20 nm in size (see also Fig. 2). Residual graphite was found even at elevated temperatures, indicating that an appreciable amount of oxygen had not diffused into the centre of the material (the TEM foils were taken from the centre of the samples). As a consequence, low high-temperature creep rates of the silicon-carbonitride-derived covalent polycrystalline composite are predicted. Moreover, the presence of graphite is consistent with the chemical composition of the polysilazane precursor. Thus the phase composition did not change on heat-treatment at 1,400 or 1,600 °C (in 0.1-MPa atmospheres of nitrogen or oxygen, respectively). However, the question why α -Si₃N₄ tends to grow during oxidation whereas α -SiC crystallizes in nanosized particles (graphite seems to be inactive during this process) is still open and under investigation.

Polysilazanes are now commercially available, and should provide suitable precursors for the synthesis of these dense Si₃N₄/SiC/C-micro/nano-composites in bulk. Their use in coatings and fibre composites should also be possible. □

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Porous clay heterostructures formed by gallery-templated synthesis

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THE mesoporous molecular sieves developed recently^{1,2} have stimulated a great deal of interest in large-pore materials for selective catalysis³⁻⁵ and other applications⁶. The synthesis of these materials involves the use of ionic surfactants which interact with the inorganic ions in a cooperative process to form a range of ordered mesostructures^{7,8}. Many of these same surfactants can be intercalated into layered inorganic materials^{9,10}, and we explore here the possibility of using intercalated surfactants for the templated synthesis of structures within the interlayer spaces (galleries) of clays. The surfactants act in concert with aqueous silicate species to form a silica framework between the layers. Removal of the surfactant by calcination then leaves a mesoporous solid with thermally stable pores of widths in the range 14–22 Å. These materials, which we call porous clay heterostructures, might provide new opportunities for the rational design of heterogeneous catalysts.

In some respects, our approach here is similar to conventional pillaring reactions of lamellar solids. But whereas pillared clays are formed by the insertion of dense, nanoscale aggregates into the galleries of the layered host, our intra-gallery templating process involves the *in situ* assembly of surfactant-inorganic precursor nanostructures, the morphology of which will be determined by the collective energetics of the inorganic and organic species as they assemble together.

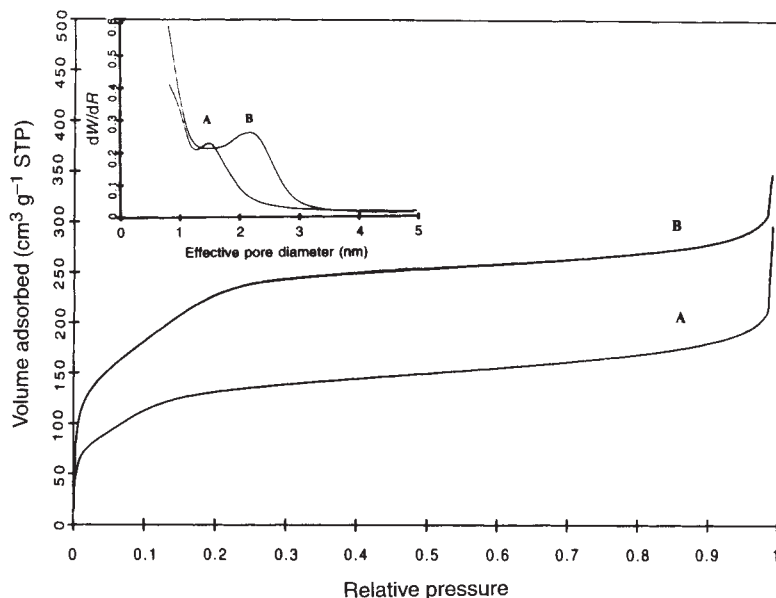
Our approach to designing porous clay heterostructures (PCHs) is based on the use of intercalated quaternary ammonium cations and neutral amines as co-surfactants to direct the interlamellar hydrolysis and condensation polymerization of neutral inorganic precursor (for example, tetraethylorthosilicate, TEOS) within the galleries of an ionic lamellar solid. High-charge-density smectite clays such as Li⁺-fluorohectorite (Li_{1.12}[Li_{1.12}Mg_{4.88}]Si₈O₂₀F₄, Corning) are well suited layered

hosts, as illustrated in the syntheses that follow. Li⁺-fluorohectorite (Li⁺-FH) was converted to a quaternary-ammonium-exchanged form (Q⁺-FH) by ion-exchange with a twofold excess of aqueous [C₁₆H₃₃N(CH₃)₃]⁺Cl[−], washing the intercalate free of excess surfactant, and air-drying. Mixtures of the hydrated Q⁺-FH (~7 H₂O per O₂₀F₄ unit cell), neutral amine and TEOS at molar ratios in the range 1:2:15 to 1:20:200 were allowed to react for 4 h at ambient temperature. Under these stoichiometric conditions the galleries are swollen by the co-templating amine and readily accessible to TEOS. Also, the extra-gallery water concentration is low, as judged by the water content of the amine (~0.4 wt%). Thus, the base-catalysed hydrolysis of TEOS is much faster in the clay galleries than in solution, and this minimizes the formation of extra-gallery silica. The resulting intercalates were centrifuged, dried in air to further promote intra-gallery TEOS hydrolysis and then calcined at 600 °C to remove the templating surfactants. Two or more orders of (001) X-ray reflections, indicating layered structures, were observed for all products. Depending on the chain length of the neutral amine, the gallery height could be varied from 14.9 Å (hexylamine) to 23.4 Å (dodecylamine). Nitrogen BET surface areas were in the range 470–750 m² g^{−1}.

Figure 1 illustrates the N₂ adsorption-desorption isotherms for the PCHs prepared from C₁₆H₃₃N(CH₃)₃⁺-FH in the presence of hexylamine and decylamine as co-templates. The nearly linear portions of the adsorption curves in the partial pressure region ~0.02–0.25 are indicative of supermicropores (~14–20 Å diameter) or small mesopores (~20–25 Å)¹¹. As shown in Fig. 1 inset, a Horvath-Kawazoe analysis¹² of the adsorption data yields average pore sizes of 15 Å (hexylamine) and 21 Å (decylamine), indicating that the neutral amine co-surfactant plays an important structure-directing role in the synthesis of the gallery-intercalated silica. On the basis of the widths at half maximum for the pore size distribution curves, the pore structures of PCH materials (~7 Å peak width) are similar in uniformity to MCM-41 mesostructures (~5 Å peak width) formed by surfactant templating². Significantly, the same average pore sizes and gallery compositions (~7.7 mol silica per O₂₀F₄ unit cell) are observed for PCH products prepared at C₁₆H₃₃N(CH₃)₃⁺-FH: octylamine: TEOS reaction stoichiometries of 1:2:15, 1:5:37.5 and 1:20:150. Also, PCH formation is not quantitative unless the TEOS: amine ratio is ≥7.5. At ratios below this value two phases are formed, namely, the surfactant-templated PCH and a silica-intercalated derivative with a basal spacing of 12.6 Å.

Table 1 gives the pore sizes of related PCHs prepared using other combinations of Q⁺ and neutral amines as co-templates.

FIG. 1 Nitrogen adsorption-desorption isotherms for porous silica-fluorohectorite clay heterostructures (PCHs) prepared by gallery-templated synthesis using (A) hexylamine and (B) decylamine as neutral co-templating surfactants. The [C₁₆H₃₃N(CH₃)₃]⁺-FH:amine:TEOS reaction stoichiometry was 1:20:150. Relative pressure is P/P_0 , where P is the equilibrium pressure of the adsorbate and P_0 is the saturation pressure of the adsorbate at the temperature of the adsorbent; the volume absorbed is at STP. Inset, the corresponding Horvath-Kawazoe pore size distribution curves (dW/dR is the derivative of the normalized adsorbate (nitrogen) volume adsorbed with respect to the pore diameter of the adsorbent). Before measurement each sample was heated overnight at 150 °C and 10^{−6} torr. The isotherms were measured at −196 °C on a Coulter Omnisorp 360CX Sorptometer using standard continuous adsorption procedures.



Increasing the chain length of the amine co-template systematically increases the pore size of the PCH. That the chain length of Q^+ also is important in the templating process is evidenced by the PCH prepared using $C_{10}H_{21}N(CH_3)_3^+$ and decylamine as gallery co-templates. Consistent with a gallery-templated pathway, this latter surfactant system affords an average pore size of 14 Å, substantially smaller than the 21-Å pore size provided by $C_{16}H_{33}N(CH_3)_3^+$ and decylamine as co-templates. Included in Table 1 are the gallery heights for the calcined PCHs, the corresponding air-dried PCH precursors, and the initial amine-solvated Q^+ -FH. The PCH gallery heights parallel the Horvath-Kawazoe pore sizes, suggesting that the pores of the gallery-templated silica are symmetrical.

The relationships between PCH pore size distributions, chain lengths of the templating surfactants, and reaction stoichiometry support a templating mechanism analogous to structure-directed M41S mesostructure syntheses^{2,13,14}. A pillaring mechanism, wherein dense laterally spaced silica aggregates are formed in the gallery, is unlikely based in part on the absence of a pore-size dependence on clay: silica ratio. Also, the observed pore sizes are uncharacteristic of pillaring. All previously reported pillared clays exhibit average pore sizes that are limited by the lateral separations of the pillars to values <10 Å (ref. 15). In contrast, PCHs exhibit pore sizes substantially larger than this pillaring limit, as well as other features indicative of templating (see below). As outlined in Fig. 2, we propose that PCH pore structures are determined solely by intrinsic structure-directing interactions between the intra-gallery surfactants and the inorganic precursor. The initial amine-solvated Q^+ -FH is assigned a lipid-like structure (Fig. 2a). This structure is substantiated by gallery heights that are very near the values expected based on the chain lengths of Q^+ and the neutral amine (see Table 1). On the introduction of TEOS, some of the neutral amine is displaced from the galleries, and a reactive intermediate is formed (Fig. 2b). Subsequent hydrolysis of the gallery TEOS affords a hydrous silica templated around a monolayer of micellar Q^+ and neutral amine assemblies. Surfactant assemblies formed exclusively from Q^+ or neutral amines are precluded by the fact that both templating components are needed for the formation of a gallery nanostructure. The reaction of Q^+ -FH alone with TEOS fails to produce a heterostructure. Spherical micelles in van der Waals contact or, more likely (based on analogies to the templating of hexagonal mesostructures, that is, MCM-41^{2,13,14}), rod-like micelles are formed, as shown in cross-section in Fig. 2c. These latter assemblies are structurally analogous to the layered silica-surfactant intercalates that have been postulated to form as intermediates in the synthesis of MCM-41¹³, with the important exception that the layers in the case of PCHs consist of pre-formed anionic 2:1 layered silicate. The final calcination step

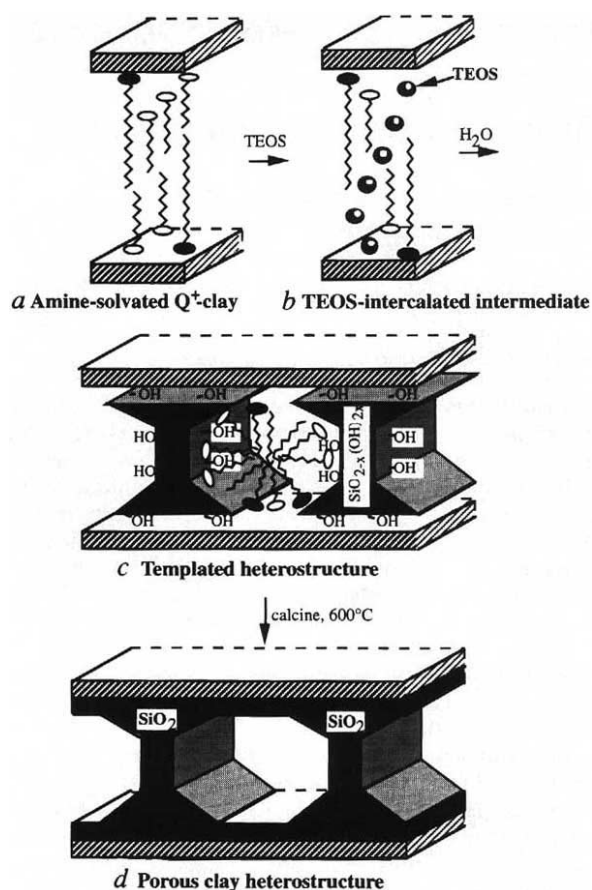


FIG. 2 Proposed mechanism for the formation of a PCH by gallery-templated synthesis. a, Amine-solvated bilayer structure with a thickness equivalent to the length of the quaternary cation (represented by filled-head groups) and the neutral amine (open-head groups) surfactants; b, intercalation of TEOS by partial displacement of neutral amine; c, interactions between the surfactants and silicate ions introduced into the interlayer space give rise to rod-like micellar assemblies of Q^+ and neutral amine surrounded by hydrated silica structures. The fraction of Q^+ in the micelle is determined by clay layer charge matching (d) Calcined porous clay heterostructure with a two-dimensional framework of porous silica intercalated between the clay layers.

(Fig. 2d) removes the template and completes the dehydroxylation and cross-linking of the gallery-assembled silica structure.

Gallery-templated syntheses of PCH materials are not limited to fluorohectorite. We also have prepared PCH derivatives of vermiculite, rectorite and magadiite by gallery-templated TEOS hydrolysis in the presence of $C_{16}H_{33}N(CH_3)_3^+$ and decyl amine as co-surfactants. Each of these PCH derivatives had an average pore size equal (within experimental uncertainty) to the pore size observed for the corresponding fluorohectorite system (~ 21 Å). This result further supports intragallery templating and unequivocally rules out pillaring. If the intra-gallery hydrolysis of TEOS simply involved the formation of dense silica pillars between the quaternary ammonium ions, the pore size should decrease with increasing charge density on the host layers. For the layered hosts investigated in the present work, the layer charge per 100 Å^2 increases in the order rectorite (1.33) < FH (2.50) < magadiite (3.27) < vermiculite (3.82). Yet, the observed pore sizes are invariant over this entire layer-charge range. This result precludes pillaring, but supports templating by micellar units comprised of neutral amines and quaternary ammonium ions in the appropriate layer charge-compensating ratio (Fig. 2).

Earlier studies have reported the intra-gallery hydrolysis of TEOS for primary-alkyl-ammonium-exchanged forms of magadiite in both the presence and the absence of primary

TABLE 1 Properties of PCHs prepared by gallery-templated synthesis

Alkylammonium exchange ion, Q^+	Amine co-template	PCH† pore size (Å)	Gallery heights* (Å)		
			Calcined PCH	Air-dried heterostructure	Amine- Q^+ -FH
$C_{16}H_{33}N(CH_3)_3^+$	$C_6H_{13}NH_2$	15	14.9	22.2	32.2 (30.5)‡
	$C_8H_{17}NH_2$	17	18.4	22.4	34.4 (33.0)
	$C_{10}H_{21}NH_2$	21	22.4	28.4	36.4 (35.5)
	$C_{12}H_{25}NH_2$	22	23.4	34.4	38.4 (38.0)
$C_{10}H_{21}N(CH_3)_3^+$	$C_{10}H_{21}NH_2$	14	14.3	23.9	29.5 (29.3)

* Gallery height is defined as the observed X-ray basal spacing minus the 9.6-Å thickness of the clay layer.

† Pore size obtained by Horvath-Kawazoe analysis of N_2 adsorption data.

‡ Values in parenthesis are the calculated gallery heights for a lipid-like bilayer of Q^+ and neutral amine based on the following estimates of chain lengths (Å): $C_{16}H_{33}N(CH_3)_3^+$, 21.5; $C_{10}H_{21}N(CH_3)_3^+$, 15.3; $C_6H_{13}NH_2$, 9.0; $C_8H_{17}NH_2$, 11.5; $C_{10}H_{21}NH_2$, 14.0; $C_{12}H_{25}NH_2$, 16.5; the observed gallery heights for $C_{16}H_{33}N(CH_3)_3^+$ and $C_{10}H_{21}N(CH_3)_3^+$ -FH were 16.5 and 12.4 Å, respectively, as expected for inclined orientations of the chains in the absence of solvating neutral amines.

amines^{16, 18}. However, these silica-intercalated magadiites have been described as pillared derivatives. We have verified that primary-ammonium-ion-exchanged forms of magadiite do indeed form pillared derivatives and not templated PCHs. For instance, octylamine-solvated octyl ammonium magadiite as a reaction precursor affords silica intercalates with 9–15 Å gallery heights depending on TEOS: magadiite ratio¹⁶. The average pore size of these derivatives (~6 Å) is substantially smaller than the gallery heights, a feature characteristic of clays pillared by dense metal oxide aggregates¹⁵. Also, the observed pore size is smaller than that expected (~12 Å) for a micelle-templated nanostructure. Thus the amine-solvated galleries of primary-ammonium-ion-exchanged forms of magadiite are accessible to TEOS for microporous pillaring, but not for nanostructure templating.

Our efforts to image PCH pores by transmission electron microscopy (TEM) have met with limited success owing to the turbostratic nature of the intercalates. For vermiculite as the layered host, the intra-gallery hydrolysis of TEOS in the presence of $C_{16}H_{33}N(CH_3)_3^+$ and decylamine as co-templates affords a calcined PCH with a gallery height of 33.3 Å. TEM images of the vermiculite heterostructure (not shown) reveal clear evidence for a lamellar morphology and uniformly expanded galleries.

PCHs bridge a potentially important pore-size region between microporous zeolites and pillared clays (<10 Å) and M41S mesostructures (>20 Å). Heavy crude oils, biological molecules (for example, metalloporphyrins) and other large macromolecular species can be accommodated in PCH galleries for shape-selective processing or catalysis. Surfactant removal from as-synthesized PCHs by calcination results in the formation of protons, which are necessary to balance the clay layer charge. Consequently, silica-interlayered PCHs are intrinsically acidic, whereas pure silica mesostructures possess little or no acidity. Initial studies indicate that calcined PCHs are capable of transferring protons from the clay layers to organic substrates adsorbed in the gallery nanopores. For instance, the acid-catalysed dehydration of 2-methyl-3-buten-2-ol can be carried out

with >99% selectivity over a silica-interlayered PCH, but no significant conversion occurs over an MCM-41 silica. Also, PCH structures are stable indefinitely, whereas MCM-41 silicas have been observed to degrade structurally on storage for only two months under ambient conditions¹⁹. Because of the complementary chemical functionality of the layered and gallery-templated components, and the stable pore size distributions in the supermicropore to small mesopore range (14–22 Å), porous heterostructures formed by gallery-templated reactions of layered silicate clays offer new opportunities for the rational design of heterogeneous catalyst systems. Also, because silicate clays are members of a larger family of ionic lamellar materials that include, for example, titanates, niobates and group IV metal phosphates, the gallery-templated syntheses demonstrated here should lead to a broad range of porous heterostructures based on diverse classes of layered hosts. □

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Climate-driven flushing of pore water in peatlands

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NORTHERN peatlands can act as either important sources or sinks for atmospheric carbon^{1,2}. It is therefore important to understand how carbon cycling in these regions will respond to a changing climate. Existing carbon balance models for peatlands assume that fluid flow and advective mass transport are negligible at depth^{3,4}, and that the effects of climate change should be essentially limited to the near-surface. Here we report the response of groundwater flow and porewater chemistry in the Glacial Lake Agassiz peatlands of northern Minnesota to the regional drought cycle. Comparison of field observations and numerical simulations indicates that climate fluctuations of short duration may temporarily reverse the vertical direction of fluid flow through the peat, although this has little effect on water chemistry⁵. On the other hand, periods of drought persisting for at least 3–5 years produce striking changes in the chemistry of the pore water. These longer-term changes in hydrology influence the flux of nutrients and dissolved

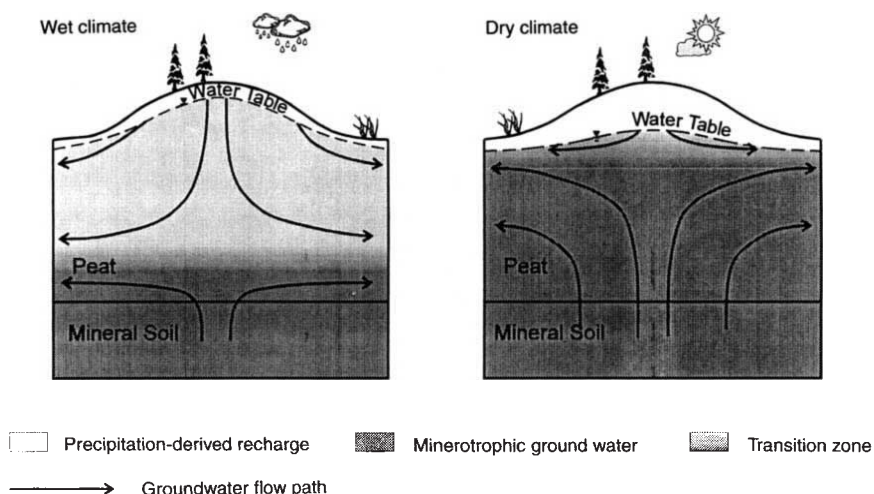
organic matter through the deeper peat, and therefore affect directly the rates of fermentation and methanogenesis, and the export of dissolved carbon compounds from the peatland.

Global climate changes should change the carbon balance of northern peats—peats that now store 150–450 Pg of carbon^{2,6} (1 Pg is 10^{15} g). Most of the carbon in these peatlands is mineralized very slowly to carbon dioxide and methane because microbial metabolism is restricted to anaerobic pathways in waterlogged and anoxic peat^{3,7,8}. If fluid flow is negligible in deeper peat, carbon mineralization will be limited by the “litter quality” of the peat itself, which becomes less degradable and more nutrient-deficient with time⁹. Climate changes would then have a greater effect on the surface layers of peatlands that are most susceptible to drying and rapid oxidation⁶.

Peatland hydrologists have long assumed that water does not flow through the deeper portions of a peat profile because the hydraulic conductivity of many peats may be too low^{10–15}. But decomposed buried wood layers, macropore openings adjacent to roots, and other separations, can locally increase the hydraulic conductivity of humified peat by several orders of magnitude¹⁶. The macro-scale hydraulic conductivity of peat may be similar to that of mineral soils, in which the greater the length of the flow path, the greater the probability that macropores will be intersected^{17,18}.

Macro-scale hydraulic conductivity may explain the close connection that exists between groundwater flow systems and porewater chemistry within the Glacial Lake Agassiz peatlands. In these extensive peatlands, water-table mounds under raised bogs drive local flow systems forcing water downwards through the peat to the underlying mineral soil¹⁹. The vertical hydraulic gra-

FIG. 1 Diagram showing changes in groundwater flow under a raised bog in the Lost River peatland during wet (left) and dry (right) years. Ground water moves from high hydraulic head to low hydraulic head, measured as the elevation of the water in piezometers. During wet years, the hydraulic head of the water-table mound is high enough to cause precipitation-derived recharge to flush mineral-rich ground water from the peat column. During persistent drought, the water-table mound dissipates and ground water in underlying mineral soils can move upwards through the peat towards the land surface.



dients of these flow systems range^{20–22} from 0.02 to 0.03. These local flow systems dissipate during seasonal and extended droughts when the water-table mounds receive insufficient precipitation to sustain them⁵. During these times, ground water upwells into the peat from underlying flow systems (Fig. 1)⁵. However, an important question remains as to whether the advective displacement of pore water can effectively transport solutes within the peat column.

During the past 15 years, we obtained hydrogeological data from the Lost River peatland of northern Minnesota to determine the response of its groundwater flow systems and porewater chemistry to a regional drought cycle. A four-year drought occurred immediately before 1981, and was followed by a seven-year wet period before an extreme drought from 1988 to 1991 (G. Spoden, personal communication). The Lost River peatland contains a 16-km² raised bog with a water-table mound ~1 m higher than the water table of the surrounding fen^{23,24}. The surface water on the bog has²⁴ a dissolved-solids content of <20 mg l⁻¹ and a pH <4. The bulk hydraulic conductivity of the 3-m of humified (haemic) peat at the bog is¹⁶ of the order of 10⁻⁴ cm s⁻¹, similar to that measured elsewhere in the Glacial Lake Agassiz peatlands⁵. Trace- and major-element profiles, surface textures of quartz silt in the peat and measurements of hydraulic head indicate that the bog is located over a discharge zone of a regional groundwater flow system^{21,25,26}. The recharge area driving the regional flow system may be a beach ridge ~10 km north of the peatland^{22,27}. Seasonal increases in the height of the water-table mound prevent the migration of minerotrophic (solute-rich and near-neutral pH) ground water into the uppermost portion of the saturated peat column (Fig. 1).

In the summers of 1982, 1983, 1990 and 1991, we measured hydraulic head and porewater chemistry from piezometer nests installed at the crest of the raised bog^{5,21}. We also constructed a simple finite-element model to simulate the transport of solutes through the peat column^{28,29}. The material properties and seepage velocities chosen for the model were based on those determined from our previous studies^{5,16}. Typical rates of vertical specific discharge are of the order of 10⁻⁷ cm s⁻¹, the effective porosity is ~0.1 (ref. 30), and dispersivity ranges³⁰ from about 4 to 10 cm. We initially assumed the extreme case that minerotrophic ground water continually discharged into the bog peat until the model calculated a steady-state profile of solute concentrations. Then the hydraulic-head gradient was reversed, and the model was run to simulate the flushing of the ground water by precipitation-driven recharge for periods of 1, 4, 8 and 10 years (Fig. 2). Identical results were obtained by running the computer simulations in reverse; that is, by initially assuming that precipitation had flushed the bog peat to steady state, and then reversing the hydraulic gradients to simulate minerotrophic groundwater discharge into the peat.

The concentration profiles calculated by the models agree with those measured in the field (Figs 2 and 3). In 1983, the pH in peat pore water increased from <4.5 in the surface water to ~6.0 until 50 cm below the peat surface; specific conductance increased from <60 to ~200 µS cm⁻¹, respectively²¹ (Fig. 3). The pH and specific-conductance profiles have marked inflection points that define the boundary between minerotrophic ground water discharging upwards into the peatland, and dilute precipitation percolating downwards from the peat surface²¹.

In contrast to 1983, pore water in 1990 had a pH of <5 and specific conductance <200 µS cm⁻¹ from the peat surface to

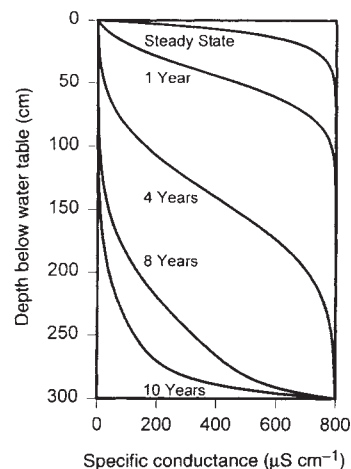
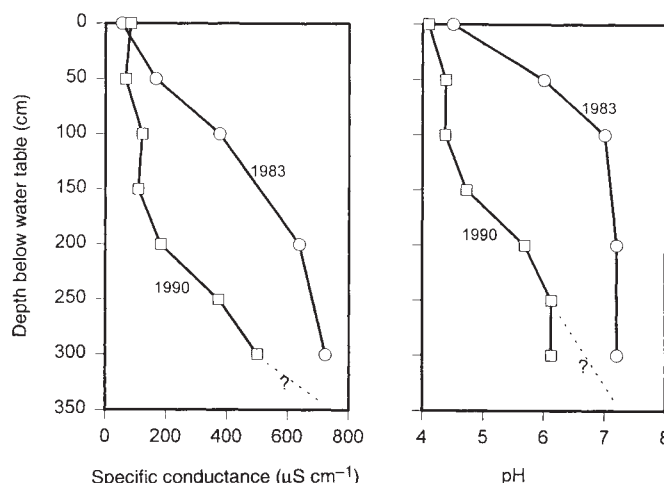


FIG. 2 Results of computer simulations, showing the advective-dispersive displacement of minerotrophic peat pore water by acidic ground water. In this simulation, it was assumed that the average linear velocity was 10⁻⁶ cm s⁻¹, and that the dispersivity was 10 cm. The shapes of the specific-conductance profiles produced by the simulations compare favourably with the shapes of the pH and specific-conductance profiles in Fig. 3, and confirm that groundwater advection governs porewater chemistry in the humified peat at Lost River peatland. The numerical model is based on the following equation¹⁷:

$$n_e \frac{\partial C}{\partial t} = n_e D \frac{\partial^2 C}{\partial Z^2} - \frac{\partial (q_z C)}{\partial Z}$$

where q_z is the vertical specific discharge, n_e is the effective (interconnected) porosity, C is the concentration of solute, D is the coefficient of hydrodynamic dispersion, and Z is the depth below water table. The finite-element method is a popular numerical technique described in a large body of literature^{28,29}. We modelled one-dimensional flow using a two-dimensional model consisting of bilinear quadrilateral elements, and considered the air-water and peat/mineral-soil interfaces as constant concentration boundaries with specific conductance values of 0 and 800 µS cm⁻¹, respectively. No flow boundaries were imposed along the sides of the model to simulate one-dimensional flow.

FIG. 3 Profiles of specific conductance (left) and pH (right) in pore water in humified bog peat, Lost River peatland. Note the dramatic decreases in values from 1983 to 1990 throughout most of the peat column. Minerotrophic pore water was almost completely replaced by acidic surface-water recharge.



almost 2.0 m depth (Fig. 3). Concentration profiles of all major solutes, including dissolved inorganic carbon, calcium, magnesium and sodium, conform to the shapes of the specific-conductance profiles^{5,21}. From 1983 to 1990, recharge waters, derived from precipitation, displaced ground water in the upper two-thirds of the peat column. Computer simulations indicate that advective-dispersive transport is responsible for observed changes in pH and specific-conductance profiles. The 1983 specific-conductance profile agrees with those produced by computer simulation within the first few years after the vertical hydraulic-head gradients reversed (in the model) from steady-state discharge ("upwelling") to recharge ("infiltration") conditions, whereas the 1990 profiles conform to those produced by longer simulated recharge (Fig. 2).

The pH profiles also generally agree with the results of the computer simulations, but their inflection points (marking the advective-dispersive front) are more pronounced than those for specific conductance. The concentration of hydrogen ions decreases at the interface where acidic pore water percolating downwards from the peat surface mixes with minerotrophic ground water upwelling from the underlying calcareous glacial deposits. At this interface, the hydrogen ions react with the alkalinity contributed to the mixture of the ground water. The consumption of hydrogen ions enhances the chemical signal at the mixing interface for ground water and surface water. The numerical simulation further shows that peat pore water at the Lost River peatland can be completely displaced by either precipitation or ground water in ~10 years, assuming that the direction and magnitude of vertical hydraulic-head gradient and material properties of the peat remain constant for that time. Sharp inflections in solute profiles, such as those observed in 1983, are produced shortly after the vertical hydraulic-head gradients reverse; the more gradual chemical profiles reflect transport over longer time periods.

The minerotrophic pore water near the land surface in summer 1983 is consistent with extensive ground water upwelling from the underlying mineral soil, although such discharge was not hydraulically measured at that time. In contrast, the acidic pore water measured in the summer of 1990 is consistent with extensive downward percolation of precipitation-derived recharge, although ground water actually moved uniformly upward throughout the peat profile⁵. The porewater chemical profiles in 1983 reflect the upwelling during the 1975–79 drought that occurred before sampling. Similarly, the porewater chemistry during the drought of 1988–91 primarily reflected the precipitation recharge that occurred during the previous seven wet years. So the chemical composition of bog pore water may not indicate the "long-term" hydrogeological state of bogs²¹, but rather a transient climate signal that occurs several years before the time of porewater sampling.

These findings have important implications for the concept of a deep-peat system (the 'catotelm' of Ingram¹⁴) with no advective movement of porewater solutes¹⁴. Groundwater flow systems, altered by climate changes, may have a major and unanticipated control over the cycling of chemicals (particularly carbon) in peatlands. During wet times when groundwater mounds drive local flow systems, nutrients and labile organic substrates (such as root exudates) are advected downwards into the deeper peat, stimulating fermentation and methanogenesis^{31–33}. Dissolved carbon compounds may also be exported from the peatland if recharge waters penetrate the underlying mineral soil²⁵. But when a discharge regime predominates, these dissolved organic compounds are flushed from the pore fluids as ground water is advected upward into the peat profile. Methanogenesis within the deeper peat is then restricted to the more refractory carbon of the peat itself. □

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Continental rifting and initial sea-floor spreading in the Woodlark basin

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OUR understanding of the processes by which continents rift and sea-floor spreading initiates is derived primarily from studies either of old passive margins and oceanic crust or of young regions of intra-continental extension where spreading has not yet started. It has been thought that continental rifting ceases when sea-floor spreading begins^{1,2}, that oceanic fracture zones develop from transfer or transform faults within continental rifts^{3,4}, and that linear magnetic anomalies correlate with the onset of sea-floor spreading during times of magnetic reversals^{5,6}. Here we present a marine geophysical survey of one of the few active examples of continental rifting and spreading initiation, the western Woodlark basin/Papuan peninsula region of New Guinea, which shows that in detail these assumptions do not hold. The data confirm models of the rifting to spreading transition that invoke both ridge propagation and nucleation of discrete spreading cells⁷⁻¹⁰, and provide an unambiguous example of a spreading centre reorienting by synchronous jumping rather than propagation.

During the past 6 Myr the formerly contiguous, eastward extensions of the Papuan peninsula (the Woodlark and Pocklington rises) were stretched, separated and subsided as the spreading centre in the Woodlark basin extended westwards between them¹¹⁻¹³ into orogenically thickened crust that is 25–50 km thick and rises 1–3 km above sea level¹⁴ (Fig. 1). Structures associated with the continental rifting include numerous

full and half grabens¹⁵, and large metamorphic core complexes on the D'Entrecasteaux islands and the Papuan peninsula^{16,17}. Normal movement along a ductile mylonitic shear zone (1–2 km thick) exposed on the islands resulted in the rapid uplift of deep metamorphic rocks (from ~30 km depth (7–11 kbar) in 4×10^6 yr) and the juxtaposition of unmetamorphosed cover rocks¹⁸⁻²¹. Granodioritic intrusion then focused uplift on several domes offset by strike-slip faults, forming topography up to 2.5 km. The extension is accompanied by peralkaline rhyolitic volcanism in the D'Entrecasteaux islands²²⁻²⁴ and by crustal tensional seismicity²⁵ as far west as 148° E. Earthquake source parameters²⁵ (Fig. 2a) indicate that low-angle (10°–25°) normal faulting is active in the region of incipient continental separation.

In April–May 1993 we conducted a geophysical and HAWAII-MR1 sidescan survey of the western Woodlark basin²⁶. The sidescan survey provided almost total-coverage bathymetry and acoustic imagery of the basin and its margins inside the bounding reefs and islands (Fig. 2a, b). Together with a magnetization map (Fig. 2c) that we derived from a three-dimensional inversion of the magnetic anomalies and bathymetry, as well as seismic reflection and gravity data shown elsewhere^{26,27}, these data provide an exceptionally clear view of the transition from intra-continental rifting to sea-floor spreading.

The V-shaped Woodlark basin shallows towards its western apex (Fig. 2a). Its neovolcanic zone, defined by the strong acoustic backscatter (Fig. 2b) within the Brunhes magnetic anomaly (Fig. 2c), extends westwards to the spreading tip at 9.8° S, 151.7° E where it abuts Moresby seamount, a crustal block that dredging²⁸ and geophysics indicates may be a metamorphic core complex. The sea-floor spreading centre in our survey area is divided into three first-order segments by the 50-km-long Moresby transform fault at 154° 12' E and by a 30-km non-transform offset at 152° 50' E (Figs 1 and 2). The first (western) segment is subdivided into three overlapping second-order segments arranged in a right-stepping *en echelon* pattern. It is characterized by numerous small seamounts and axial ridges with

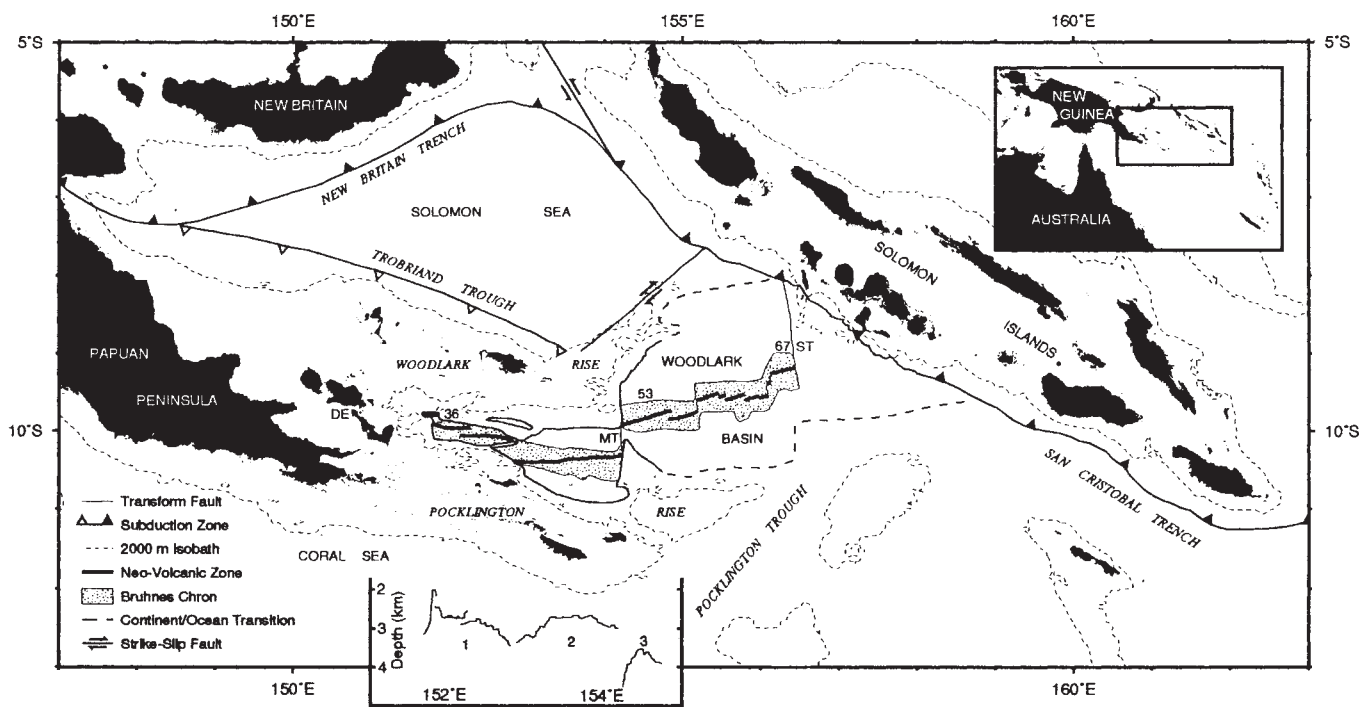
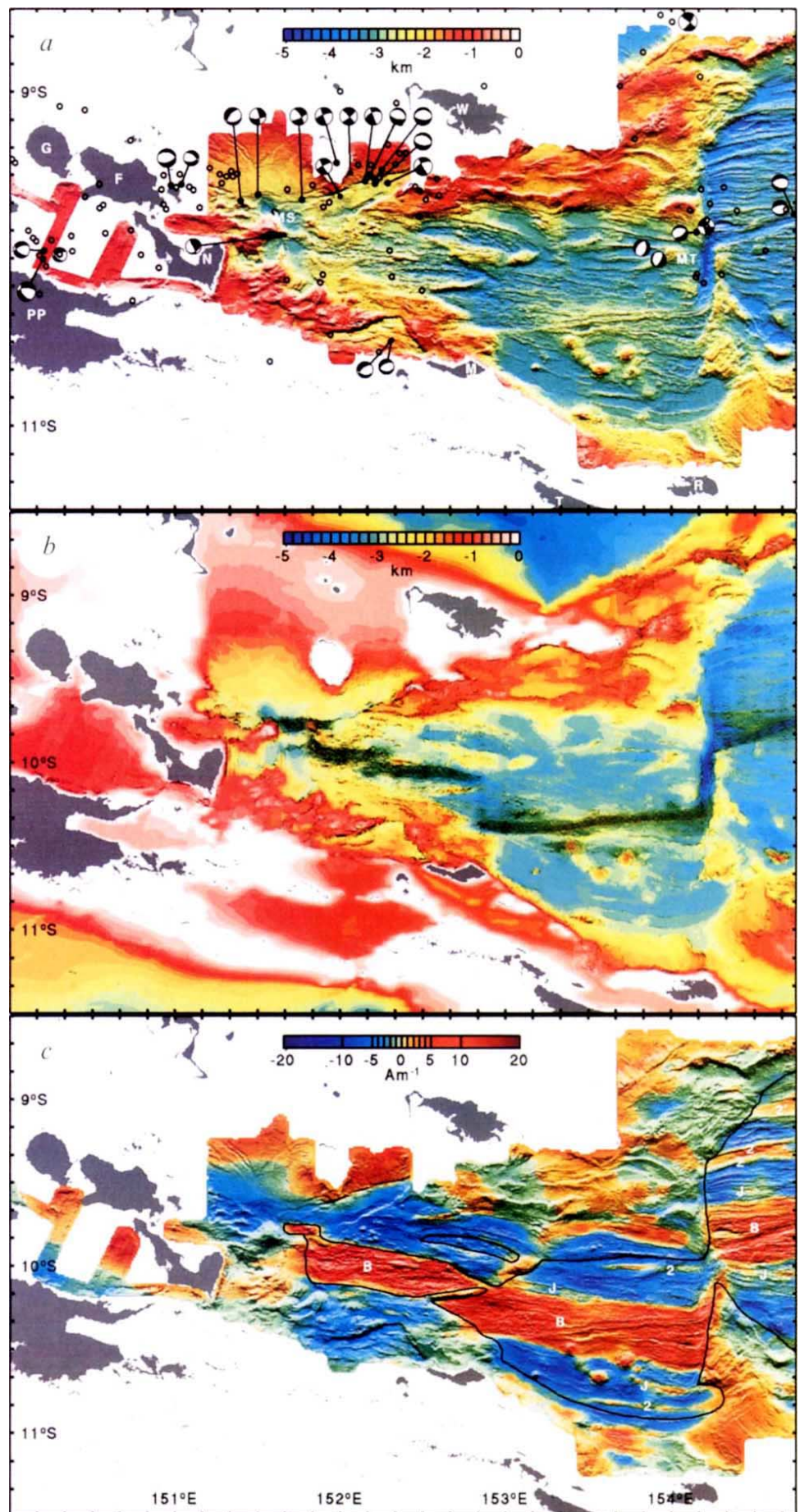
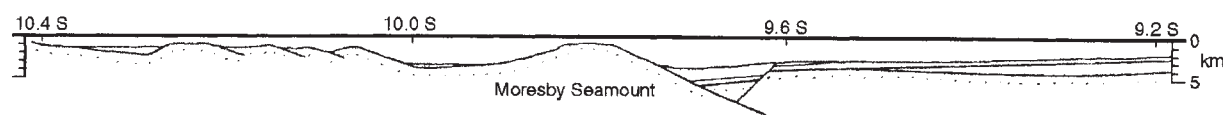


FIG. 1 Major physiographical features and active plate boundaries in the Papua New Guinea–Solomon Islands region. The stippled area encloses oceanic crust formed during the Brunhes chron, at spreading rates labelled in mm yr⁻¹. MT and ST, Moresby and Simbo transform

faults, respectively; DE, D'Entrecasteaux islands. Top inset, geographical location of study area. Bottom inset, depth profile along the axis of the Woodlark basin spreading centre, with the three first-order spreading segments numbered.

FIG. 2 Geophysical data for the western Woodlark basin. *a*, HAWAII-MR1 bathymetry, sunlit from the north, together with shallow earthquakes (small circles) located by the International Seismological Centre and earthquake focal mechanisms. PP, Papuan peninsula; G, Goodenough island; F, Fergusson island; N, Normanby island; R, Rossel island; MS, Moresby seamount; MT, Moresby transform fault. *b*, HAWAII-MR1 acoustic imagery overlain on bathymetry. *c*, Sea-floor magnetization and crustal fabric. Magnetization lineations corresponding to the Bruhnes (B), Jaramillo (J) and magnetic anomalies 2 and 2' are labelled and the landward boundary of oceanic crust is outlined. The line spacing for the survey was 5 nautical miles, adequate to achieve complete coverage of the sidescan acoustic imagery and bathymetry as well as to interpolate the magnetic anomaly field.



FIG. 3 True-scale meridional section through Moresby seamount^{27,29}.

low relief. In cross-section, the second (central) segment has a 3–4-km-wide rift valley within which there is a small axial ridge. It is subdivided by a third-order (1 km) left-stepping, non-transform offset at 153° 30' E. The third (eastern) segment, like others further east, trends ENE and has an axial valley that is 6–7 km wide and 3.5–4.0 km deep. In along-axis profile, each first-order spreading segment is shallowest in the centre and asymmetrically deepest at the ends (Fig. 1). Several young off-axis seamounts occur south of the centre of the second segment (Fig. 2). The oceanic crust has a characteristic abyssal hill fabric, with numerous superposed seamounts of varying sizes and on- as well as off-axis origins. Notwithstanding complexities in the spreading history, sea floor along a given fabric element is isochronous. For example, adjacent to segment two, magnetic anomaly 2 corresponds to an abyssal hill on both sides of the axis (Fig. 2c).

A strong seismic reflector, correlated to the low-angle fault inferred from earthquake studies, dips 25° N and has an emergent segment along the northern flank of Moresby seamount^{27,29} (Fig. 3). Basement fault blocks overlain by only minor ponded sediments constitute the southern margin of the basin WNW of Misima island. In contrast, the northern margin (above the low-angle fault) has a down-flexed pre-rift sedimentary basin and basement sequence, unconformably overlapped by syn-rift sediments fed south by submarine channels, and cut by high-angle faults with a zig-zag pattern in plan view²⁷ (Figs 2 and 3). Seismic reflection data indicate a remarkably sharp (<5 km wide at the surface) transition from oceanic crust to rifted crust in most areas^{27,29}, as has been observed in some other regions^{3,30}. There are no dipping reflector sequences indicative of excessive lava production and high degrees of mantle partial melting. (There are, however, numerous small volcanoes, a few kilometres in diameter, often erupted along margin faults.) Indeed, basalts from segment one have Na₂O contents (3.1 wt% at 8.0 wt% MgO) and other geochemical characteristics³¹ indicative of low degrees of partial melting. This area would be classified 'non-volcanic' in the two-part division of passive margin types^{32,33}.

To test the applicability of various kinematic models of spreading propagation into continental lithosphere^{7–10,34}, we have used the distinctive sea-floor fabric and seismic-reflection characteristics of oceanic versus rifted continental crust to determine the boundary between them. This boundary was derived independently of magnetization information, but is plotted on the sea-floor magnetization map (Fig. 2c) for comparison. Note that the landward boundary of oceanic crust and the magnetization contours are locally discordant. The distribution of oceanic crust reveals a segment due south of Woodlark island near 9° 50' S that is surrounded by rifted crust. Like the westernmost neovolcanic subsegment, this isolated oceanic segment is direct evidence for nucleation of discrete spreading cells (and subsequent ridge jumping) within stretched continental crust^{9,10}. In other cases, the rifting-to-spreading transition involves ridge propagation^{7–9}. Evidence includes the V-shaped margins (pseudofaults) east of Moresby transform, against which oceanic crust of successively younger age abuts (Fig. 2c). A second example is the western V-shaped tip of the Brunhes anomaly for segment two: the propagator sliced off a sliver of rifted crust that is now nearly surrounded by oceanic crust. We do not find evidence for periodically spaced spreading centres and 'punctiform' spreading initiation³⁴. Our data are best fitted by a combined spreading-centre nucleation and propagation model⁹.

The Moresby transform fault at 154.2° E is the only transform offset of the spreading axis west of 155° E. It formed just before 1 Myr and the Jaramillo magnetic anomaly. Significantly, the transform does not extend to the southern continental margin and the aulacogen northeast of Rossel island (Fig. 2). Rather, it linked offset spreading segments that, at the time of magnetic anomaly 2 (~1.9 Myr), were overlapping but separated by rifted continental crust. The oceanic structural geometry was not inherited directly from the earlier rift geometry³⁵. The initial oceanic crust in the western Woodlark basin has no transform faults and the one transform that did develop, after $\sim 0.9 \times 10^6$ yr of spreading, cut through intervening rifted crust to link overlapping spreading segments.

The ability of the offset spreading segments to open initially without forming connecting transform faults seems to be related to the fact that rifting of the conjugate margins continues after spreading has started between them. We infer from two observations that extension does not immediately localize at the ridge axis, but instead is distributed between margin rifting and sea-floor spreading. The first observation is that spreading on segment one overlaps in space and time with seismogenic faulting on the continental margins west of 153° E (Fig. 2a). The second is that the sea-floor fabric and magnetic anomalies that curve towards the spreading axis on both sides of segment three (Fig. 2) require non-rigid reconstructions of the adjacent margins, that is, that spreading propagation into continental crust is accommodated by progressively less crustal extension, instead of by the progressive cessation of spreading on an adjacent segment as in oceanic crust^{7–9}. The overlap in time between initiation of spreading and cessation of rifting along the margin can be estimated from both sets of observations to be $(0.8–1) \times 10^6$ yr. Spreading initiated west of 153° E by the beginning of the Brunhes epoch (0.78 Myr) and seismogenic faulting of the margins continues today. Adjacent to segment three, the age difference between curved and linear conjugate sea-floor fabrics at a given longitude is $\sim 10^6$ yr.

We recognize several instances where curvilinear fault systems up to 100 km long are associated with magnetization boundaries landward of oceanic crust, particularly along the northern margin (Fig. 2c). Somewhat similar magnetic anomalies have been observed in rifted arc crust of the northern Mariana trough where their formation was ascribed to fault-controlled intrusions³⁶. An alternative explanation, applicable in regions of low-angle detachments such as the Woodlark basin, is that the magnetization contrasts could form between hanging-wall rocks and footwall rocks that acquire a magnetization as they are exhumed from mid-crustal levels and cool through their Curie temperature. Both explanations provide mechanisms for magnetic lineations to form landwards of the oldest oceanic crust. Therefore caution must be exercised in attempting to locate the limits of old oceanic crust where the basement structure and crustal fabric are poorly imaged beneath sediment cover.

Our survey also provides clear evidence that a spreading centre can reorient by synchronous jumping rather than propagation. All segments other than the westernmost subsegment reoriented, as shown by their present trend being oblique to the Brunhes/Matuyama crustal boundary and sea-floor fabric within it (Fig. 2). A subsequent survey³⁷ confirmed the existence of similarly rotated spreading segments in the rest of the eastern Woodlark basin (Fig. 1). The 500-km-long Woodlark basin spreading

axis changed orientation simultaneously, not by propagation, ~100 kyr ago. In addition to the gravitational collapse of orogenically overthickened lithosphere, causes for the opening of the Woodlark basin have been sought in the 'pull' of the Solomon Sea lithosphere which is being both subducted to the north at the New Britain trench and dragged northwestwards by the over-riding Pacific plate^{11,13}. Recent changes in the resultant of these additive forces, associated with spreading ridge and arc-continent collisions to the east and north respectively^{38,39}, may be responsible for the ridge reorientation. □

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A common neural code for frequency- and amplitude-modulated sounds

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MOST naturally occurring sounds are modulated in amplitude or frequency; important examples include animal vocalizations and species-specific communication signals in mammals, insects, reptiles, birds and amphibians^{1–9}. Deciphering the information from amplitude-modulated (AM) sounds is a well-understood process, requiring a phase locking of primary auditory afferents to the modulation envelopes^{10–12}. The mechanism for decoding frequency modulation (FM) is not as clear because the FM envelope is flat (Fig. 1). One biological solution is to monitor amplitude fluctuations in frequency-tuned cochlear filters as the instantaneous frequency of the FM sweeps through the passband of these filters. This view postulates an FM-to-AM transduction whereby a change in frequency is transmitted as a change in amplitude^{13,14}. This is an appealing idea because, if such transduction occurs early in the auditory pathway, it provides a neurally economical solution to how the auditory system encodes these important sounds. Here we illustrate that an FM and AM sound must be transformed into a common neural code in the brain stem. Observers can accurately determine if the phase of an FM presented to one ear is leading or lagging, by only a fraction of a millisecond, the phase of an AM presented to the other ear. A single intracranial image is perceived, the spatial position of which is a function of this phase difference.

In experiment 1 (Fig. 1) we led an FM sound to one ear and an AM to the other ear. Both sounds had the same sinusoidal

modulation rate and carrier frequency. We imposed an interaural phase difference on the periodicities of modulation. The observer's task was to detect this phase disparity in a two-alternative, forced-choice task. Near-perfect discrimination is achieved when the FM is delayed by 2 ms, that is, half the modulation cycle. Performance is at chance (0.5 probability) when the FM is advanced by 4 ms, a full cycle of modulation. Observers report the percept of a single, intracranial image which is laterally displaced toward one ear. As the interaural phase difference exceeds 2 ms (π radians), the image's spatial position reverses towards the other ear, consistent with the iterative nature of periodic signals.

In experiment 2 we considered the properties of the envelope after transduction. For each cycle of modulation, the FM will pass twice through the central passband of a filter centred near the FM carrier: once for each direction of frequency sweep. The resultant AM will therefore have an envelope rate that is twice the FM rate. However, filters which are centred away from the FM carrier will produce AM at the same rate as the FM. For example, the FM may only sweep through the lower skirt of a filter centred above the carrier. There are many such filters both above and below the carrier frequency, and we therefore refer to this scheme as a global model. If the auditory system relies primarily on local information from filters centred near the carrier then it may miscode the modulation information. Figure 2 shows performance as a function of the FM carrier frequency and modulation rate with the AM carrier and rate fixed at 3 kHz and 250 Hz, respectively. The yellow pyramid shows equivalent FM and AM parameters. The red pyramids show equal rates but different carriers. Dark blue shows an FM rate equal to half the AM rate; good performance for dark blue supports a local model, whereas good performance for red and yellow suggests an avoidance of such misinformation in favour of using global information. Perhaps more striking is that interaural-delay sensitivity is maintained across very different carriers (red pyramids), further supporting a global model.

In experiment 3 an illusion of motion was successfully induced by linearly changing (sweeping) high-frequency tones at different rates in the two ears. Unidirectional spectral sweeps are abund-

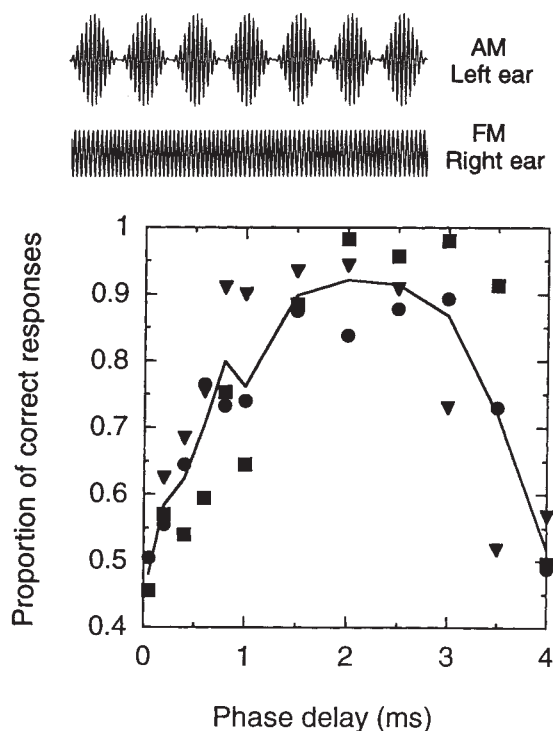


FIG. 1 Stimuli and results of experiment 1. The left ear received the AM signal and the right ear the FM. Ordinate shows proportion of correct detection of an interaural delay in a two-alternative, forced-choice task. Peak performance is observed when the 250-Hz sinusoidal modulators are antiphasic at the two ears (2 ms). Chance performance is 0.5. Different symbols represent different observers.

METHODS. Both FM and AM signals had a carrier of 3 kHz and a sinusoidal modulation rate of 250 Hz. Signals were 300 ms in duration with 20-ms cosine-squared ramps; the ramps were not interaurally delayed. Signals were digitally generated and presented at a sampling rate of 100 kHz and a level of 60 dB (2×10^{-5} N m $^{-2}$) through digital-to-analog converters and Sennheiser (HD-450) headphones in a soundproof chamber. The AM had a 100% depth of modulation and the FM index of modulation was unity. Experimental controls included intense low-pass noise (1.5 kHz cutoff; $N_0 = 30$ dB); highpass filtering (1.5 kHz); or using signals with very low sound-pressure levels (30 dB). The Sennheiser headphone transfer function was flat within 0.8 dB from 2750 to 3250 Hz. Digital inverse filtering was used to eliminate this small variation in level.

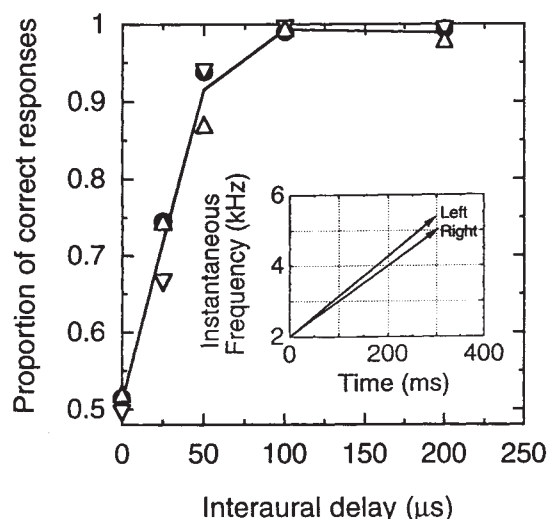


FIG. 3 Results of experiment 3. The inset shows stimuli used to induce the illusion of motion by spectrally sweeping tones in the two ears at different rates. The moving auditory image had a trajectory towards the ear which carried the faster sweep. Because motion is a subjective phenomenon, to validate these results psychophysically, the data in the figure were collected for the special case where the sweeps at the two ears had the same spectral velocity (equal time-frequency slopes). This produced a spatially stationary image. The subject's task was to detect an interaural delay in the sweep in a two-alternative, forced-choice task. Chance performance is 0.5. The solid line shows mean performance. Each symbol represents data from one observer.

METHODS. The signal was a tone of 300 ms which spectrally swept from 2 to 5 kHz. The entire waveform, except for the gating envelopes, was delayed to one ear. To obscure pitch cues, the starting frequency (y-intercept) and the slope of the frequency sweep were independently randomized on each observation by 10%. The interaural frequency difference at 2 kHz produced by delaying the sweep by 50 μ s is 1.5 Hz. Control tests showed that this cue was uninformative. The spectrum of the signal was symmetric about the sweep's terminal frequency. The amplitude of the 2–5 kHz sweep was down by 60 dB at 1.5 kHz.

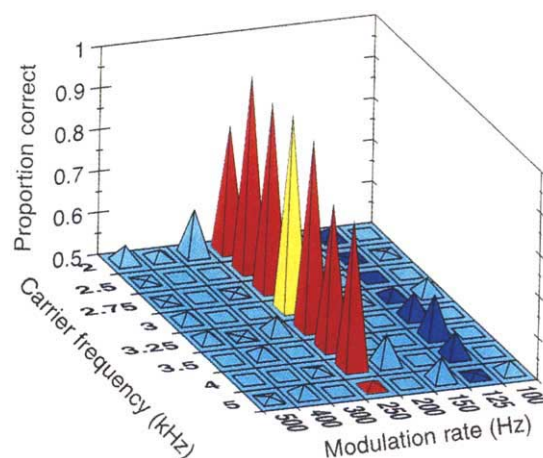


FIG. 2 Results of experiment 2. These data show interaural-delay sensitivity for unequal carriers and modulation rates. The left ear received a sinusoidal AM with a carrier of 3 kHz and a modulation rate of 250 Hz. The right ear received a sinusoidal FM, the carrier and modulation rate of which were parameters (x and y coordinates). Data were collected for all 64 combinations averaged across three observers. The yellow pyramid shows data for equivalent FM and AM rates and carriers. Red shows equivalent modulation rates but different carriers. Dark blue shows an FM rate half that of AM (see text). Methods were the same as Fig. 1.

ant naturalistic stimuli; the sonar emissions of the FM bat¹ and primate warning cells¹⁵ are two examples. Recent behavioural¹⁶ and physiological¹⁷ observations provide evidence of a monaural code for directional sweeps, but not a binaural code. It has also been known for over a century^{18,19} that binaural temporal cues are uninformative in high-frequency pure (unmodulated) tones. Tones, however, are unnatural stimuli and we reasoned that, even at high frequencies, a more natural sound created by spectrally sweeping the tone may generate impulsive rise-and-decay activity in successive, tonotopically ordered cochlear filters. A more rapid sweep in one ear would produce, relative to the other ear, correspondingly earlier neural activity in units tuned to similar frequency regions. In fact, observers reported a very strong illusion of intracranial spatial motion towards the ear that carried the faster sweep. In support of this subjective report, Fig. 3 shows binaural sensitivity to very small delays, in the order of only tens of microseconds.

The coding of information in modulated form extends to other sensory domains, for example, vision. Psychophysical experiments have illustrated visual sensitivity to AM and FM spatial frequencies^{20–22}. Physiological studies have also identified units in areas 17 and 18 of the Cat visual cortex which respond to envelope information at high spatial frequencies²³. Our results suggest that the auditory system uses a common neural code for both FM and AM information at relatively early neural stages, possibly before binaural convergence at the brain stem^{24–26}. Units responsive to monaural frequency sweeps have previously been identified; some entrain to very high rates in the range in which we have reported excellent binaural sensitivity^{17,27,28}. Although the binaural properties of such neurons have not been examined at any level of the auditory pathway, the results reported here encourage such a search. □

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Lifetime of the P-selectin-carbohydrate bond and its response to tensile force in hydrodynamic flow

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SELECTINS tether to the blood vessel wall leukocytes that are flowing in the bloodstream and support subsequent labile rolling interactions as the leukocytes are subjected to hydrodynamic drag forces^{1,2}. To support this rolling, selectins have been proposed to have rapid bond association and dissociation rate constants, and special mechanical properties linking tensile forces and bond dissociation^{3–6}. We have visualized transient tethering and release of neutrophils in hydrodynamic flow on lipid bilayers containing densities of P-selectin below those required to support rolling. We report here that transient tethers had first-order kinetics and other characteristics suggesting a unimolecular interaction between P-selectin and its glycoprotein ligand (PSGL-1). The unstressed dissociation constant (off rate) was 1 s^{-1} . Hydrodynamic shear stresses of up to 1.1 dyn cm^{-2} , corresponding to a force on the bond of up to 110 pN, increased the off rate only modestly, to 3.5 s^{-1} . The data was adequately matched by a proposed equation⁷ relating off rate to the exponential of tensile force on the bond and the bond interaction distance, and gave a bond interaction distance of 0.5 Å. This distance is compatible with hydrogen and metal coordination bonds between P-selectin and PSGL-1. Fast on and off rates, together with the high tensile strength of the selectin bond, appear necessary to support rolling at physiological shear stresses.

We used video microscopy to visualize neutrophil adhesion in shear flow to P-selectin reconstituted in lipid bilayers in a parallel-wall flow chamber. At densities from 400 to 30 sites per μm^2

of P-selectin, neutrophils in hydrodynamic flow tethered to the bilayer and then rolled across it with a jerky motion. During rolling, cells paused for short periods of time, then translated forward for a distance of less than one cell diameter, perhaps reflecting dissociation of one and retention of other selectin-carbohydrate bonds (cell 3, Fig. 1a, b). At 15 or fewer P-selectin sites per μm^2 , the jerkiness increased and periods during which the cells tethered or rolled were separated by periods during which the cell moved forward at the hydrodynamic velocity, the velocity predicted for a non-adherent cell in flow near the wall (Fig. 1a, cells 1 and 2)^{8,9}. Tethering events were defined as transient when they were separated by at least 50 μm of motion at the hydrodynamic velocity, and when no rolling ($<1 \mu\text{m}$ displacement) occurred while the cell was tethered. At 15 sites per μm^2 , 67% of the tethering events were transient, and at 6, 5, 3 and 1 sites per μm^2 , all tethering events were transient. The frequency of transient tethering was linearly related to P-selectin site density (Fig. 2). Tethering was highly specific because it was inhibited by pretreatment of the bilayers by monoclonal antibody to P-selectin, pretreatment of neutrophils with *O*-glycoprotease, or addition of EDTA (Fig. 2). This agrees with findings that the ligand for P-selectin on human neutrophils is a sialyl Lewis^x-like structure that is displayed by the mucin-like P-selectin glycoprotein ligand (PSGL-1), which is *O*-glycoprotease-sensitive, and that ligand binding requires Ca^{2+} (refs 10–12).

To determine the kinetics for release of tethered neutrophils from P-selectin bilayers (the cellular off rate) the duration of tethering events at a wall shear stress of 0.36 dyn cm^{-2} was measured for P-selectin densities from 1 to 30 sites per μm^2 (Fig. 3a). Each set of data from 1 to 15 sites per μm^2 fit a single straight line, demonstrating first-order kinetics. Furthermore, the cellular dissociation rate constant k_{off} was unaffected by P-selectin density from 1 to 15 sites per μm^2 (Fig. 3a, b). Although there may be heterogeneity in the structure of carbohydrate ligands for P-selectin displayed on PSGL-1¹², our finding of a single dissociation rate constant suggests the ligands are homogenous at least with respect to dissociation rate, and agrees with homogenous affinity¹³.

The independence of cellular dissociation rate on P-selectin density at 15 sites per μm^2 and below, the lack of rolling, and the linear rather than higher-order dependence of tethering frequency on selectin density, suggest that a quantal tethering unit has been identified. The data are consistent with the hypothesis that this quantal tethering unit is a monovalent bond between a single PSGL-1 molecule on the neutrophil and a single P-

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selectin molecule on the lipid bilayer; however, PSGL-1^{10,11} and P-selectin are dimers¹³, raising the feasibility of dimeric interactions. A single bond dissociation model was favoured by a variety of statistical tests, including Pearson's linear correlation coefficient, and examination of the χ^2 deviation by both q (ref. 14) and F -tests^{15,16}, for P-selectin densities ≤ 5 sites per μm^2 . By contrast, a two-bond dissociation model gave better fits for most data sets with P-selectin densities ≥ 15 sites per μm^2 , suggesting multivalent tethering.

Tensile force on non-covalent bonds between receptors and ligands is predicted to increase the rate of dissociation^{4,7}. The magnitude of this effect, which is particularly relevant for understanding the biophysics of selectin-dependent rolling^{5,6}, has not previously been measured in any system. The k_{off} was similar at shear stresses of 0.17 and 0.36 dyn cm^{-2} , but was consistently faster at 0.73 dyn cm^{-2} (Figs 3b and 4a). Examination of a wider range of wall shear stresses revealed that at 1.1 dyn cm^{-2} , the k_{off} increased to 3.5 s^{-1} (Fig. 4b). Using Goldman's solution for the hydrodynamic force on a sphere near a wall⁸, and approxi-

ating the lever arm between the P-selectin bond and the centre of the neutrophil as 4 μm (Fig. 4c and legend), give a tensile force of 112 pN on the bond at 1.1 dyn cm^{-2} . Bell⁷ proposed that the dissociation rate constant is related to the force on a bond, F_b , by the equation $k_{\text{off}} = k_{\text{off}}^0 \exp(\sigma F_b / kT)$, where k_{off}^0 is the dissociation rate in the absence of applied force, σ is the distance over which separation of the receptor and ligand weakens their interaction, k is Boltzmann's constant and T is temperature. A fit of this equation to our data (Fig. 4b) yields $k_{\text{off}}^0 = 0.95 \pm 0.17 \text{ s}^{-1}$ and $\sigma = 0.49 \pm 0.08 \text{ \AA}$. Our value of σ is probably accurate to a factor of 2; the main uncertainty derives from the estimate of the lever arm and hence F_b . The σ of 0.5 $\text{\AA}$ is consistent with hydrogen and calcium coordination bonds between the selectin and carbohydrate ligand^{17,18}; the variation in length of hydrogen bonds seen in crystal structures is of the same magnitude¹⁹. By contrast, hydrophobic bonds depend on the exclusion of water molecules at binding interfaces and are estimated to have a σ of the same order as the diameter of a water molecule of 3 $\text{\AA}$ (ref. 20). σ is also linked by deformation of the interface on a stressed molecule to the lateral dimensions of the binding interface and a small σ may be favoured by a smaller recognition surface for selectins^{17,18} than for typical hydrophobic protein-protein interactions²¹. The modest coupling between force and dissociation rate that we have found is ideal for maintenance of the rolling adhesion between the leukocyte and the vessel wall, because k_{off} increases only moderately at shear stresses in the physiological range (3.5-fold at 1.1 dyn cm^{-2}). Thus, the P-selectin-carbohydrate bond has high tensile strength. By contrast, with $\sigma = 3 \text{ \AA}$ for hydrophobic bonds that provide the binding energy for most protein-protein interactions, there would be a 1,000-fold increase in k_{off} at 1.1 dyn cm^{-2} according to Bell's expression; such fast dissociation clearly would greatly lower the probability of formation of a second receptor-ligand bond and would prevent rolling interactions.

The average force required to pluck a membrane protein from a cell by extracting its transmembrane domain from the bilayer is estimated to be 20 pN^{22,23}. We hypothesize that the cytoplasmic domains of selectins and their counter-receptors, such as PSGL-1, anchor these molecules to the cytoskeleton so that they can resist extraction at forces up to 112 pN; the cytoplasmic domain

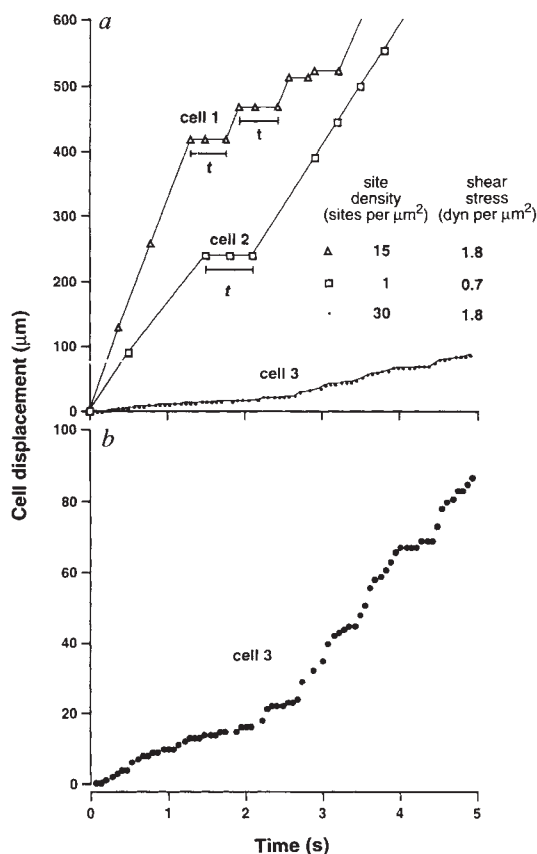


FIG. 1 Displacement in the direction of flow of representative neutrophils interacting with purified P-selectin in the parallel-wall chamber. *a*, Three individual neutrophils. Duration of transient tethers is marked (t). *b*, Enlargement of the displacement of cell 3.

METHODS. Purified P-selectin was incorporated in phosphatidylcholine bilayers on glass slides that were assembled in a parallel-wall flow chamber and mounted on an inverted phase-contrast microscope³. Selectin site density was determined and neutrophils were prepared and infused as previously described^{3,28}. Images from a Nikon plan $\times 10$ objective and a TEC-470 CCD video camera (Optronics, Goleta, CA) that yielded a 0.43 mm^2 field of view 0.76 mm in the direction of flow and 0.57 mm wide were recorded and played back on Hitachi time lapse TLC 2051 or Hi8 recorders. Playback at slow motion or frame by frame with positions of cells measured on transparencies on the monitor, or frame grabbing with a SCION LG-3 on a Macintosh Quadra 650 and analysis with NIH Image 1.55 and a routine for following motion of cells (E. Finger), were used to track neutrophils. Coordinates of cell centres were determined within one pixel accuracy ($\pm 0.7 \mu\text{m}$).

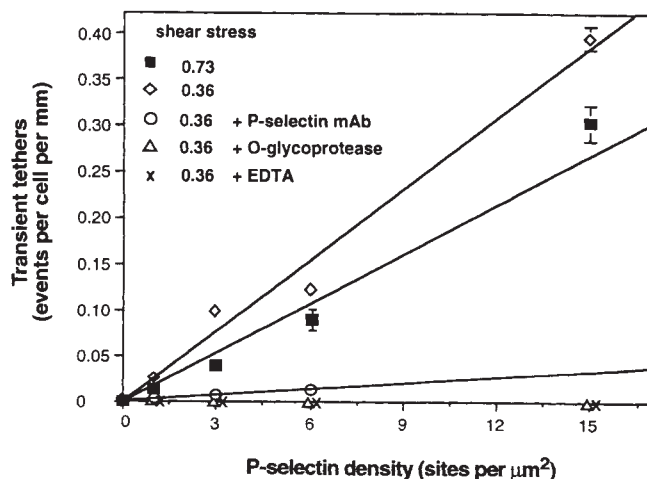


FIG. 2 Frequency of transient tethering of neutrophils is proportional to selectin site density. Using methods described in Fig. 1, the number of transient tethers was counted and divided by the number of cells that flowed across the field that were within the focal plane of the bilayer and by the path length (0.76 mm). For inhibition studies, bilayers were pretreated with $20 \mu\text{g ml}^{-1}$ G1 mAb to P-selectin at 37°C ²⁹, and tethering measured in absence of mAb; neutrophils were pretreated with $50 \mu\text{g ml}^{-1}$ O-glycoprotease, a gift from A. Mellors (University of Guelph, Ontario) for 30 min³⁰; or 5 mM EDTA was present in the assay.

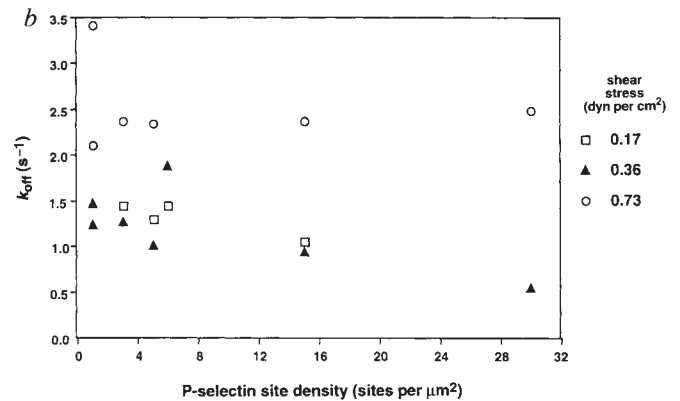
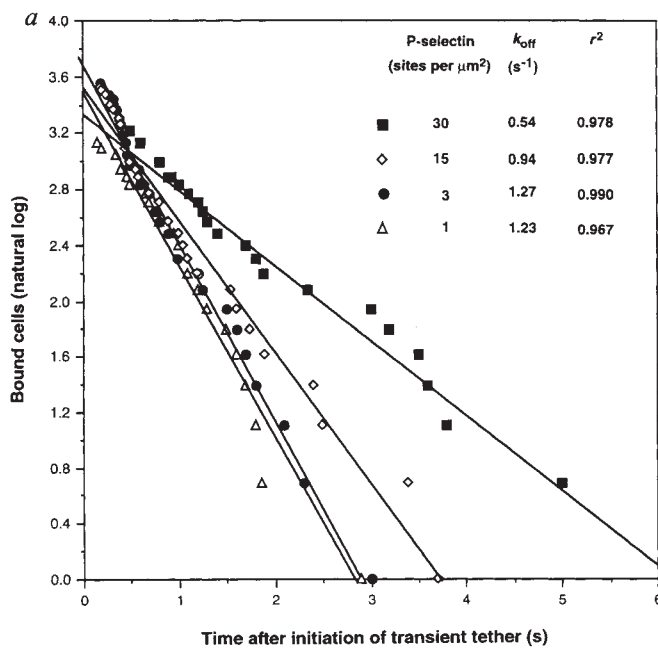


FIG. 3 Kinetics of dissociation of transiently tethered neutrophils from P-selectin in lipid bilayers. *a*, Transient tethering at 0.36 dyn cm^{-1} on bilayers containing the indicated density of P-selectin. *b*, Dissociation rate as a function of P-selectin density and shear stress. The duration of transient tethers as defined in the text was measured as described in Fig. 1. Sufficient videotape was analysed (0.5 to 3 min) to obtain 30 to 35 tethering events, and the $\ln$ of the number of cells that remained bound as a function of time after initiation of individual tethering events was plotted. The slope $= -k_{\text{off}}$. The experimental error of arrest duration measurements was two video frames ($\pm 0.07 \text{ s}$).

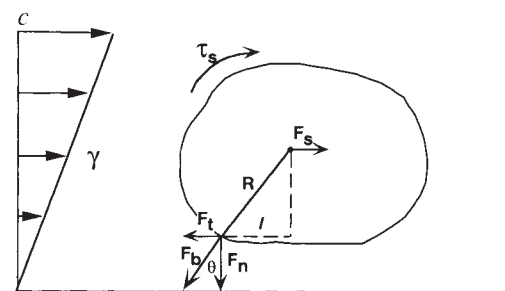
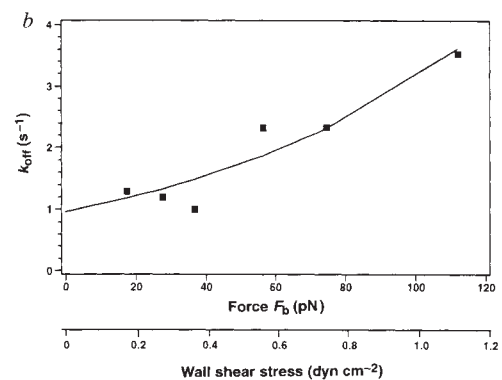
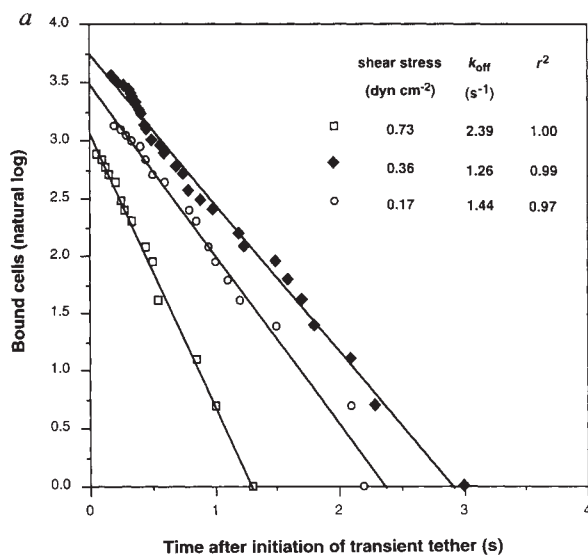
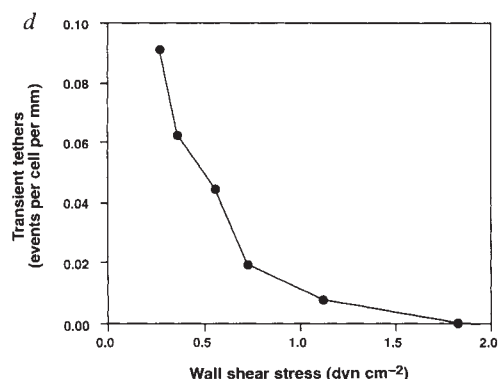


FIG. 4 Effect of the wall shear stress on kinetics of neutrophil dissociation from P-selectin bilayers and on the frequency of transient tethers. Kinetics of dissociation was measured from bilayers containing 3 sites per μm^2 (*a*) or 5 sites per μm^2 (*b*, ■) as described in Fig. 3. Line in *b* shows fit of data to expression $k_{\text{off}} = k_{\text{off}}^0 \exp(\sigma F_b / kT)$. ($k_{\text{off}}^0 = 0.95 \pm 0.17 \text{ s}^{-1}$, $kT/\sigma = 83 \pm 14 \text{ pN}$, $\chi^2 = 0.484$, $\sigma = 0.49 \pm 0.08 \text{ Å}$ at $T = 298 \text{ K}$.) *c*, Force balance on a tethered neutrophil in shear flow. Shear flow of shear rate γ will induce an external force F_s , and external torque, τ_s on a cell of radius R . The bond, which is oriented at an angle θ with the substrate, exerts a force F_b on the cell. The connection between the bond and the cell is located at a distance l from the projection of the centre of mass; the distance l is a function of the deformation of the cell and the magnitude of the force F_b , but is of the order of the cell radius. For adhesion, a cell must be in a static equilibrium: its net force and torque must be zero. Given l , this gives $F_b \cos \theta = F_s$ and $F_b l \sin \theta = \tau_s + R F_s$. Solution of these two equations with $l = 4 \mu\text{m}$ and $R = 4.25 \mu\text{m}$ gives $\theta = 55.5^\circ$. A shear stress of 1.1 dyn cm^{-2} gives $F_b = 112 \text{ pN}$, and there is a linear relationship between shear stress and F_b . If a smaller value of l is assumed, θ increases, as does F_b at a given shear stress. *d*, Transient tethers at 5 sites per μm^2 were measured as described in Fig. 2.



and an intact cytoskeleton are required for rolling on L-selectin²⁴. At 1.8 dyn cm⁻² or 183 pN and above, we did not detect tethering at low P-selectin densities (Fig. 4d), which may reflect extraction from the membrane. Extraction does not appear to occur during rolling³, which at high P-selectin densities occurs up to 35 dyn cm⁻² (ref. 3), corresponding to a force of 3,500 pN; maintenance of rolling may require that this force be distributed over multiple selectin-ligand bonds.

High on and off rates have been proposed to be important for selectin-mediated rolling³. Using the $K_a = 1.4 \times 10^7 \text{ M}^{-1}$ for binding of truncated, monomeric P-selectin to neutrophils¹³ and our measured value of $k_{\text{off}} = 0.95 \text{ s}^{-1}$, $k_{\text{on}} = 1.5 \times 10^7 \text{ s}^{-1}$. Both k_{off} and k_{on} are indeed fast compared to other tabulated macromolecular interactions²⁵. The CD2-LFA-3 interaction has a 40-fold slower on-rate but 6-fold faster off-rate²⁶ and does not mediate rolling²⁷. However, interaction of a cell-bound monoclonal antibody with dinitrophenol does not mediate rolling despite a k_{on} twofold faster and a k_{off} only threefold slower than P-selectin⁹. We suspect the antibody-dinitrophenol interaction fails to support rolling because it depends on hydrophobic bonds with lower tensile strength, or because of extraction from the membrane. Thus, not only fast on and off rates, but also high tensile strength of the receptor-ligand bond and anchoring of the receptor and ligand to the cytoskeleton may be important for rolling. Although not all adhesion molecules support rolling, other adhesion molecules including the integrins and cadherins bear tensile force. These molecules may be distinguished from adhesion molecules that function primarily in signalling, and from receptors for soluble ligands. Common features may have been selected in the binding sites of selectins, integrins and

cadherins, including a requirement for divalent cations, that allow them to resist tensile stress. □

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Eating disorder and epilepsy in mice lacking 5-HT_{2C} serotonin receptors

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SEROTONIN (5-hydroxytryptamine, 5-HT) is a monoaminergic neurotransmitter that is believed to modulate numerous sensory, motor and behavioural processes in the mammalian nervous system¹⁻³. These diverse responses are elicited through the activation of a large family of receptor subtypes⁴. The complexity of this signalling system and the paucity of selective drugs have made it difficult to define specific roles for 5-HT receptor subtypes, or to determine how serotonergic drugs modulate mood and behaviour. To address these issues, we have generated mutant mice lacking functional 5-HT_{2C} receptors (previously termed 5-HT_{1C}), prominent G-protein-coupled receptors that are widely expressed throughout the brain and spinal cord and which have been proposed to mediate numerous central nervous system (CNS) actions of serotonin^{3,5-8}. Here we show that 5-HT_{2C} receptor-deficient mice are overweight as a result of abnormal control of feeding behav-

iour, establishing a role for this receptor in the serotonergic control of appetite. Mutant animals are also prone to spontaneous death from seizures, suggesting that 5-HT_{2C} receptors mediate tonic inhibition of neuronal network excitability.

Mice lacking 5-HT_{2C} receptors (5-HT_{2C}R) were generated by introducing a nonsense mutation into exon 5 of the cognate gene, thereby placing a stop codon within the fifth putative transmembrane segment of the receptor and eliminating the carboxy-terminal half of the protein (Fig. 1a). When this mutation was introduced into the corresponding position of the rat 5-HT_{2C}R complementary DNA, the resultant cRNA failed to express functional receptors in *Xenopus* oocytes (not shown). The 5-HT_{2C}R gene is X-linked^{9,10}, and targeted J1 and D3 male (XY) embryonic stem cells therefore showed disruption of a single allele (Fig. 1b). Brains of hemizygous mutant male mice (-/-) were shown to lack 5-HT_{2C}Rs by two independent tests. First, immunocytochemical analysis of coronal sections from brains of wild-type animals using a 5-HT_{2C}R antiserum revealed staining along the apical surface of the choroid plexus epithelium, where 5-HT_{2C}Rs are abundantly expressed^{5,8} (Fig. 1c). In contrast, sections from mutant animals were devoid of immunoreactivity, even though the choroid plexus exhibited normal morphology and expression of transthyretin, a cytoplasmic protein that serves as a marker for this tissue¹¹. Second, an electrophysiological assay in *Xenopus* oocytes showed that brains of mutant animals lack functional 5-HT_{2C}R transcripts (Fig. 1d).

Initial inspection of the CNS of 5-HT_{2C}R-deficient animals revealed no gross anatomical or functional abnormalities. No aberrations in ventricular size or brain cytoarchitecture were observed by examination of Nissl-stained sections throughout the neuraxis. Hippocampal long-term potentiation, a form of neural plasticity, was similar when measured in the CA1 and CA3 regions of slice preparations from brains of mutant and wild-type animals (not shown). We also examined the sensitivity of these animals to nociceptive stimuli because the distribution

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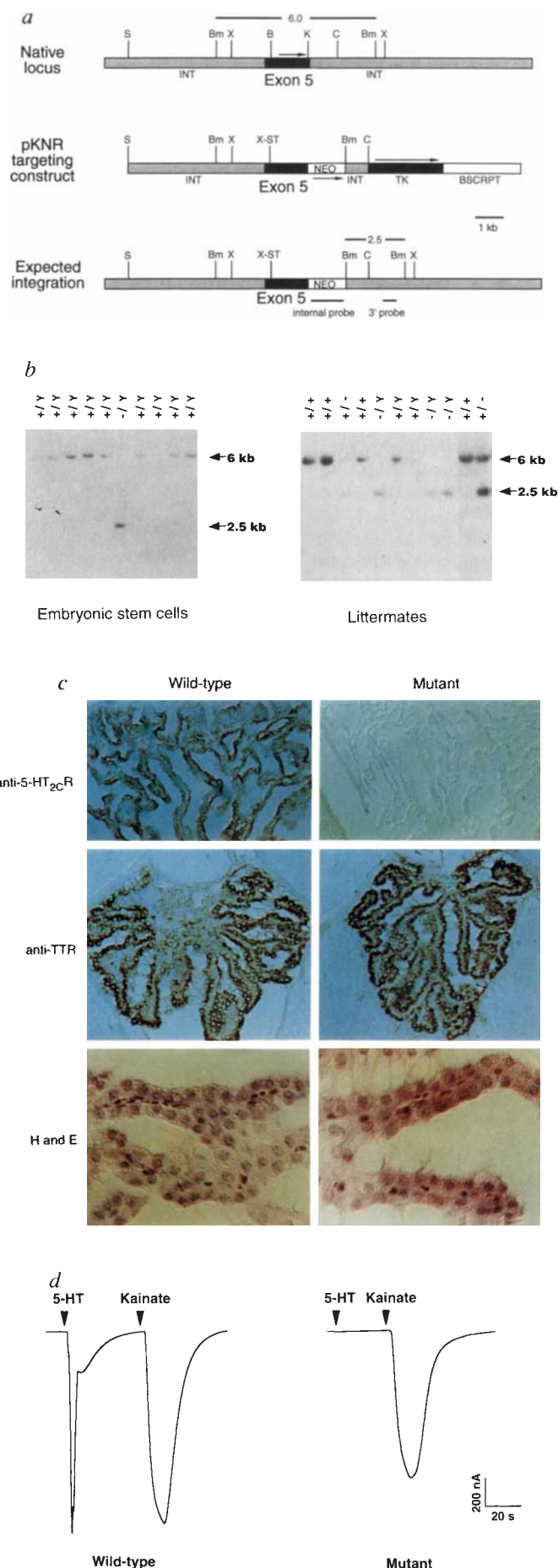


FIG. 1 Targeted mutation of the 5-HT_{2c} receptor gene. **a**, Schematic representation of a portion of the 5-HT_{2c}R gene, pKNR targeting construct, and expected targeted allele. To create a nonsense mutation, a 16-base-pair (bp) oligonucleotide (CTAGTCTAGACTAGCC) containing stop codons in three reading frames and an XbaI recognition site (X-ST) was inserted into the BsmI site (B) of exon 5. A neomycin resistance cassette (NEO) was inserted into the KpnI(K) site of the exon. The mutated fragment was cloned into the pGK-TK plasmid, which contains the HSV thymidine kinase gene driven by the PGK promoter (TK) in a Bluescript (BSCRPT) vector (Stratagene). Locations of 3' flanking and internal probes for Southern blot analysis are indicated. **b**, Southern blot analysis of BamHI (Bm)-digested genomic DNA from double drug selected embryonic stem cell transfectants, and from tails of littermates produced from crossing a female heterozygous mouse with a wild-type male. Blots were hybridized with a 3' flanking probe. Wild-type and mutant alleles correspond to 6.0-kb and 2.5-kb fragments, respectively. **c**, Immunocytochemical analysis using an antibody to the C-terminal tail of the 5-HT_{2c}R revealed staining along the apical membrane of the choroid plexus epithelium of wild-type, but not mutant, mice. Choroid plexus of mutant and wild-type mice were indistinguishable when stained with antibody to transthyretin (anti-TTR) or with haematoxylin and eosin (H and E). **d**, Whole-cell voltage-clamp recordings revealed a marked inward current following bath application of serotonin to *Xenopus* oocytes injected with poly(A)⁺ RNA from brains of wild-type mice. No detectable current was observed with mRNA from mutant siblings. As a positive control, application of kainate, a glutamate receptor agonist, evoked inward currents of similar magnitude in both cases. **METHODS.** An 8.5-kb DNA fragment containing exon 5 of the 5-HT_{2c}R gene was isolated from a 129/Sv mouse genomic library and used to construct the pKNR targeting vector. J1 or D3 embryonic stem cells were electroporated with linearized pKNR and a positive-negative selection procedure²⁷ was used to enrich for targeted clones. Surviving embryonic stem cell colonies were screened for homologous recombination events by Southern blot analysis and targeted clones were obtained in D3 and J1 cells with an overall frequency of 1/45. Male chimaeras produced by injection of these cells into C57B1/6J blastocysts were bred with B6D2 mice, and germline transmission of the mutation was achieved with independent clones derived from both D3 and J1 parental lines. Female heterozygotes were mated to wild-type siblings or C57B1/6J males to produce hemizygous mutant males bearing a single disrupted allele of the 5-HT_{2c}R gene. Expected distributions of gender and targeted mutation were observed among progeny. For immunocytochemistry, an affinity-purified polyclonal antiserum was raised against a synthetic peptide (CVVSEISSV) corresponding to the C-terminal tail of the 5-HT_{2c}R. Brains of mutant and wild-type littermates were fixed and sectioned (13 µm thickness) as described²⁸. Immunostaining was performed using an avidin-biotinylated peroxidase complex (ABC kit, Vector Laboratories). Messenger RNA preparation and oocyte electrophysiology were carried out as described²⁹. Oocytes were injected with 50 ng poly(A)⁺ RNA from the brains of wild-type or mutant male siblings. Responses were evoked with 10-s pulses (arrow heads) of serotonin (10 µM) or kainate (100 µM), at a holding potential of -80 mV.

of 5-HT_{2C}Rs suggested that they participate in pain regulation⁶. Nevertheless, mutant and wild-type animals did not differ in their sensitivities to thermal or mechanical stimulation (not shown).

Despite the lack of an overt anatomical or physiological phenotype, time revealed that a significant fraction of mutant animals died prematurely (Fig. 2a). Deaths were not preceded by any noticeable health problems, such as weight loss, dehydration or discomfort, and autopsies showed no evidence of haemorrhage, infarction or ischaemia that might be associated with cardiovascular failure or stroke. However, continuous videotape monitoring of small groups of mice revealed the occurrence of spontaneous epileptic seizures that were often preceded by repetitive grooming of the snout. This motor activity appeared to generalize so that the mouse would fall on its side and progress to seizures characterized by repetitive hyperkinetic (tonic-clonic) movements. In most cases, the mouse quickly recovered and resumed apparently normal behaviour. This cycle might then be repeated over the next few minutes. Such episodes occurred sporadically with a frequency not greater than two or three per day. Occasionally seizures progressed to a tonic extension phase, leading to respiratory arrest and death within seconds of seizure onset.

To provide a more quantitative comparison of relative seizure susceptibilities in mutant and wild-type animals, we used a pharmacological assay in which mice were infused intravenously with the epileptogenic GABA_A receptor antagonist pentamethylenetetrazole (metrazol). Metrazol infusion elicits a series of stereotyped responses beginning with a period of intermittent twitches of the head and body, leading to tonic-clonic activity, followed by a phase of tonic extension and death¹². The time (or cumulative drug dose) required for mutant and wild-type animals to progress from one phase of this response to the next was monitored in a blinded fashion, and several major differences were seen (Fig. 2b). Mutants showed a 24% reduction in seizure threshold (onset of first twitch response), an 83% reduction in the duration of the tonic-clonic phase, and a 48% reduction in the lethal dose, relative to wild-type controls. These results suggest that the loss of 5-HT_{2C}R function leads to both a lowered

TABLE 1 Behavioural eating disorder revealed by restricted feeding analysis

		Fed <i>ad libitum</i>	Pair-fed and fasted
Body mass (g)	Wild type	32.5 ± 1.0 (18)	23.4 ± 0.9 (9)
	Mutant	36.1 ± 1.6 (21)	22.2 ± 0.8 (9)
Body length (mm)	Wild type	95.0 ± 0.8 (8)	86.9 ± 0.8 (9)
	Mutant	95.6 ± 1.6 (9)	86.0 ± 0.8 (9)
Plasma glucose (mg dl ⁻¹)	Wild type	145 ± 9 (14)	143 ± 11 (9)
	Mutant	144 ± 8 (15)	160 ± 11 (9)
Plasma insulin (ng ml ⁻¹)	Wild type	3.4 ± 1.5 (14)	1.2 ± 0.3 (9)
	Mutant	6.9 ± 3.5 (15)	1.5 ± 0.7 (9)

Body weights and lengths, and glucose and insulin levels were determined for wild-type and 5-HT_{2C} receptor-mutant mice that had been fed *ad libitum* (ages 11–15 weeks; see Fig. 3a), or pair-fed for 4 weeks postweaning and fasted overnight before morning sample collection. Mean ± s.e.m. (numbers of mice). Values representing significant differences between wild-type and mutant animals ($P \leq 0.01$) in bold. For paired feeding studies, nine pairs of individually housed mice, each consisting of one mutant and one wild-type littermate, were established at the time of weaning (3 weeks). Wild-type animals had *ad libitum* access to food, whereas each day mutant animals were provided only the amount of food consumed by their wild-type partners. Values were determined as in Fig. 3.

seizure threshold and a more rapid progression of seizure activity. To determine whether this difference between mutant and wild-type animals can be mimicked in normal animals by pharmacological manipulation, we administered metrazol to C57B1/6J mice that had been pretreated with 1-(3-chlorophenyl)piperazine (mCPP), a non-selective 5-HT_{2C}R agonist, or with mesulergine, a non-selective antagonist. The duration of the tonic-clonic phase of the seizure response was significantly greater in animals pretreated with agonist compared with those pretreated with antagonist (Fig. 2c). This result supports the hypothesis that the epileptic phenotype exhibited by 5-HT_{2C}R-deficient mice does not arise from indirect developmental or compensatory changes in the brain resulting from loss

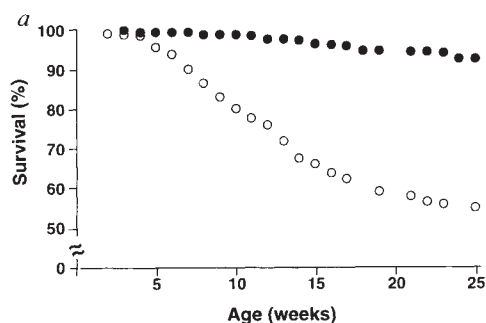
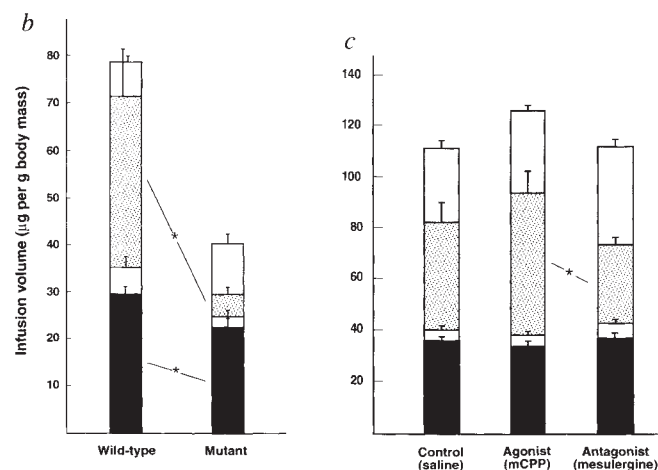


FIG. 2 Mice lacking 5-HT_{2C} receptors are prone to sudden death from seizures. a, Spontaneous deaths among hemizygous mutant male mice were first seen in the fifth postnatal week. Filled and open circles represent wild-type and mutant animals, respectively. b, Seizure threshold and latency following the timed intravenous infusion of pentamethylenetetrazole (metrazol) are reduced in mutant male mice compared with wild-type littermates. Wild-type ($n=10$) and mutant ($n=13$) male mice (11–15 weeks old) received a continuous infusion of metrazol into the tail vein at a constant rate. The time required for the animals to progress from one phase of the seizure response to the next was monitored. Data were corrected for body weight and converted to the corresponding metrazol dose. Stages include latency to first twitch (black), isolated twitches of the head and body (white), hyperkinetic tonic-clonic seizures (stippled), and tonic extension (grey). Values represent mean ± s.e.m. * $P \leq 0.005$. c, The tonic-clonic phase of a metrazol-induced seizure in normal mice is extended by pretreating with a serotonergic antagonist relative to pretreatment with agonist. C57B1/6J



mice were administered metrazol 30 min after intraperitoneal injection with agonist (mCPP; 5 mg per kg) ($n=10$), antagonist (mesulergine; 2 mg per kg) ($n=11$), or saline (vehicle), ($n=14$). Values represent mean ± s.e.m. * $P \leq 0.02$.

METHODS. Mice were loosely held in a plexiglass restrainer and administered a continuous infusion of 0.5% metrazol in phosphate buffered saline into the tail vein at a constant rate of 0.36 ml min⁻¹. Experimenters were blinded to the genotype of the animal and the drug administered.

of 5-HT_{2C}R activity, but instead reflects a normal adult function of this receptor to suppress neuronal network excitability.

Serotonergic drugs are known to modulate appetite^{3,13,14}, so we were intrigued by the observation that mutant mice were often heavier than their normal littermates. In rare instances, mutants grew to be twice as heavy as their wild-type siblings, attaining masses of up to 82 g. To determine quantitatively whether the loss of 5-HT_{2C}R resulted in increased body mass, seven litters, each consisting of at least two hemizygous mutant males and two wild-type males, were used for analysis of body and organ masses (Fig. 3a). We found a significant (13%) increase in average total body mass of mutant animals compared with wild-type siblings, with no difference in body length. Mutant animals also had larger fat stores, as exemplified by a 48% increase in the deposition of perirenal white adipose tissue. Brown adipose tissue, which is primarily involved in thermoregulation, was not significantly altered in mutant animals.

Some non-selective serotonergic agonists with relatively high affinities for 5-HT_{2C}Rs (mCPP, for example) act as appetite suppressants^{15,17}. To determine whether this effect is specifically

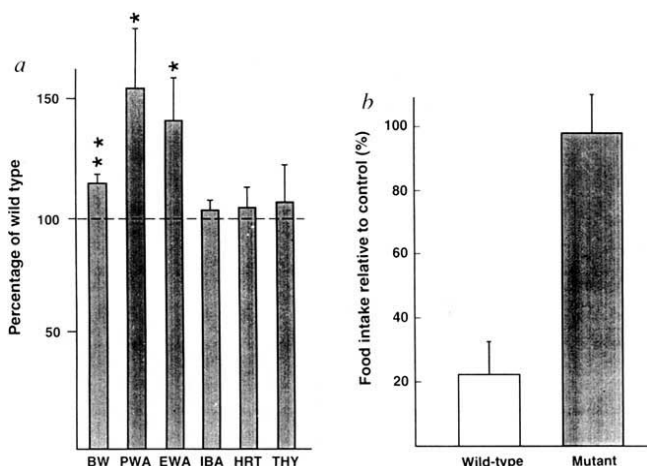


FIG. 3 Mutant mice are overweight and do not respond to an appetite suppressant drug. **a**, Mutant mice showed significantly greater body weight (BW), perirenal white adipose tissue (PWA) and epididymal white adipose tissue (EWA) deposits compared with wild-type siblings. Interscapular brown adipose tissue (IBA), heart (HRT) and thymus (THY) weights did not differ significantly between wild-type and mutant animals. Mean values for mutant animals of each litter are expressed as a percentage of the values for wild-type littermates. Values represent mean $\pm$ s.e.m. * $P \leq 0.05$; ** $P \leq 0.01$. **b**, 5-HT_{2C}R-deficient mice do not respond to the appetite suppressant actions of the serotonergic agonist mCPP. Food consumption was measured in wild-type and mutant animals after administration of mCPP. For each animal, values were normalized to separate 'sham' trials in which only saline was administered before feeding. Administration of mCPP drastically reduced food intake by wild-type animals, but not by 5-HT_{2C}R mutants. Values represent mean $\pm$ s.e.m. $P \leq 0.001$.

METHODS. Adult mice (11–15 weeks old) from seven litters were killed early in the light cycle, and tissue and blood samples were collected for the determination of weights, uncoupling protein content of brown adipose tissue, glucose and insulin values. All measurements were made as previously described³⁰, except that insulin levels were determined using a Linco radioimmunoassay kit (St Charles, Missouri). For mCPP trials, mutant ($n=13$) and wild-type ($n=12$) male mice were administered (in blinded fashion) either mCPP (5 mg per kg intraperitoneally) or control solution (saline) following an 18-h overnight fast. Food was provided 30 min after injection, and consumption over the ensuing 2-h period was measured. One week later, the procedure was repeated, except that mice that received mCPP on the previous week received control solution, and vice versa. Each mouse therefore served as its own baseline control. Food consumed following mCPP administration is expressed as percentage of consumption following administration of control solution.

mediated through activation of 5-HT_{2C}Rs, we compared the appetite-suppressant actions of mCPP on wild-type and mutant animals. Whereas mCPP drastically reduced food intake by wild-type mice (78%), there was no effect on the amount of food consumed by mutant animals (Fig. 3b), demonstrating that the 5-HT_{2C}R is the primary site at which this non-selective serotonergic drug exerts its appetite-suppressant action.

Two types of mechanism have been proposed to underlie obesity in various rodent models, namely, metabolic defects in which there is an increased predisposition to store calories in white adipose fat depots, and behavioural abnormalities that lead to increased food consumption in the setting of a normal metabolic state. These two mechanisms can be distinguished by a standard paired feeding analysis¹⁸. At the time of weaning, mutant animals were paired with wild-type littermates. Wild-type animals had *ad libitum* access to food, whereas each mutant animal was given only the amount of food consumed by its wild-type partner. After 4 weeks, animals were analysed for body mass and length and their organs weighed. If the overweight phenotype is due to a metabolic defect, then calories will be stored in fat depots at the expense of linear growth. As a result, mutant animals would be shorter than their wild-type littermates and have elevated fat stores. In the case of a behavioural abnormality, restricted feeding would normalize body composition of mutant animals relative to their paired wild-type littermates. We found the latter to be the case because body mass, length and white adipose tissue depots of pair-fed mutants were not significantly different from wild-type controls, nor were plasma levels of glucose and insulin (Table 1). This contrasts with the *ad libitum* situation, in which mutant animals were heavier than their wild-type siblings, but of the same length (Fig. 3a; Table 1). The elevated variance in insulin levels of mutant animals fed *ad libitum* probably reflects variations in the interval between eating and the collection of serum samples. This is verified by the normalization of insulin levels following an overnight fast (Table 1), and contrasts with the elevated insulin levels commonly seen in rodent metabolic obesity syndromes¹⁸. Finally, levels of brown adipose tissue uncoupling protein, an indicator of sympathetic nervous system activity, were normal in mutant animals fed *ad libitum* (not shown). Decreased sympathetic nervous system drive to brown adipose tissue frequently accompanies metabolic obesity¹⁹.

The distribution of 5-HT_{2C}R and the actions of non-selective drugs have prompted speculation that this serotonin receptor subtype participates in processing and integration of sensory information, regulation of central monoaminergic systems, and modulation of neuroendocrine responses, anxiety, feeding behaviour and cerebrospinal fluid production^{3,6–8}. The unexpected observation of an epileptic phenotype in mice devoid of 5-HT_{2C}R now implicates this subtype in the modulation of neuronal network excitability, consistent with previous observations that changes in serotonergic systems can alter the threshold for seizure activity^{20–23}. Within CNS neurons, highest levels of 5-HT_{2C}R transcripts are found in the hippocampus^{6,7}, a temporal-lobe structure that is a common focus for seizures involving stereotyped or repetitive movements²⁴ such as those reported here. Regardless of the location of the seizure focus, this genetic model has several features in common with some forms of human epilepsies: the onset of seizure episodes is spontaneous and not readily inducible and the occurrence is infrequent and sporadic²⁴. Decreased 5-HT_{2C}R activity may represent a mechanism for seizure genesis that provides a rationale for the development of anticonvulsant agents.

Our studies also demonstrate that the 5-HT_{2C}R is important in the serotonergic control of feeding and appetite, and that diminished serotonergic signalling in 5-HT_{2C}R mutant mice leads to increased food intake because of a behavioural disorder rather than a metabolic abnormality. Indeed, drugs such as fluoxetine (Prozac) and dexfenfluramine, which enhance serotonergic transmission by blocking transmitter re-uptake, control

overeating associated with certain obesity syndromes¹⁴. Moreover, 5-HT_{2C}R transcripts have been detected in the paraventricular nucleus of the hypothalamus⁷, lesions of which result in a behavioural obesity syndrome¹⁸. Thus, 5-HT_{2C}R mutant mice differ from other rodent models of obesity such as *ob/ob* and *db/db* mice^{25,26}, which are characterized primarily by metabolic dysregulation. 5-HT_{2C}R deficient mice therefore provide a novel tool for elucidating defects in neurochemical pathways that underlie the abnormal control of appetite observed in mood and eating disorders. □

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CpG motifs in bacterial DNA trigger direct B-cell activation

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UNMETHYLATED CpG dinucleotides are more frequent in the genomes of bacteria and viruses than of vertebrates. We report here that bacterial DNA and synthetic oligodeoxynucleotides containing unmethylated CpG dinucleotides induce murine B cells to proliferate and secrete immunoglobulin *in vitro* and *in vivo*. This activation is enhanced by simultaneous signals delivered through the antigen receptor. Optimal B-cell activation requires a DNA motif in which an unmethylated CpG dinucleotide is flanked by two 5' purines and two 3' pyrimidines. Oligodeoxynucleotides containing this CpG motif induce more than 95% of all spleen B cells to enter the cell cycle. These data suggest a possible evolutionary link between immune defence based on the recognition of microbial DNA and the phenomenon of 'CpG suppression' in vertebrates. The potent immune activation by CpG oligonucleotides has implications for the design and interpretation of studies using 'antisense' oligonucleotides and points to possible new applications as adjuvants.

In addition to antigen-specific immune defences, lymphocytes, phagocytes and the complement system are directly activated by several microbial products including formyl methionine, teichoic acid, peptidoglycan, exopolysaccharides and lipopolysaccharide (LPS) (reviewed in ref. 1). Bacterial DNA also induces murine B-cell proliferation and immunoglobulin production, whereas mammalian DNA does not (Fig. 1a and ref. 2). In considering explanations for the mitogenicity of bacterial DNA, we noted that CpG dinucleotides generally are present at the expected

frequency of 1 per 16 dinucleotides in microbial DNA, but they are only about one-quarter as prevalent in vertebrates³. In addition to this 'CpG suppression', the cytosines in CpG dinucleotides are highly methylated in vertebrates, but not microorganisms³.

Methylation of bacterial DNA with CpG methylase abolished mitogenicity, demonstrating that this difference in CpG status is the cause of B-cell stimulation by bacterial DNA (Fig. 1a). The stimulatory effects of bacterial DNA were reproduced with synthetic oligodeoxynucleotides (oligonucleotides) that contain one or more CpG dinucleotides (Table 1). B cells isolated by flow cytometry to greater than 99% purity showed greater or equal activation to that represented in Table 1, demonstrating that accessory cells were not required. Oligonucleotides lost stimulatory activity if the CpG was eliminated (Table 1; compare oligonucleotide 1 to 1a; 2 to 2a, 3D to 3Dc; and 3M to 3Ma); if the oligonucleotide length was reduced below 8 bases (Table 1, oligonucleotide 2 versus 2e); or if the cytosine of the CpG was replaced by 5-methylcytosine (Table 1; oligonucleotides 1b, 3Dd and 3Mb). In contrast, methylation of other cytosines (oligonucleotides 3De and 3Mc), or of CpG close to the end of an oligonucleotide (oligonucleotide 1c), did not reduce activity.

Optimal B-cell activation was seen with oligonucleotides containing a CpG motif in which the CpG dinucleotide was flanked by two 5' purines (preferably a GpA dinucleotide) and two 3' pyrimidines (preferably a TpC or TpT dinucleotide). Oligonucleotides in which bases were switched to bring the CpG motif closer to this ideal showed improved stimulation (for example, oligonucleotide 3M versus 3Md) whereas substitutions that disturbed the motif reduced stimulation (for example, compare oligonucleotide 2 to 2b, 2c and 2d or 3D to 3Df). Substitutions outside this CpG motif had little effect on stimulation (compare oligonucleotide 1 to 1d; 3D to 3Dg; 3M to 3Me). These data confirm that a CpG motif is the essential element present in oligonucleotides that activate B cells.

Kinetic studies demonstrated that B-cell activation was detectable by 4 h, peaked at 24 h, and returned toward baseline by 48 h (Fig. 1b). Cell-cycle analysis demonstrated that CpG oligonucleotides induce more than 95% of B cells to enter the cell cycle; a greater percentage than that induced by optimal LPS concentrations (Table 2). We also found that CpG oligonucleotides stimulate natural killer (NK) activity and protect B-cell

lines against anti-IgM-induced growth inhibition (A.M.K. *et al.*, manuscripts in preparation).

CpG oligonucleotides with a nuclease-resistant phosphorothioate-modified backbone were synthesized to determine whether they would also cause immune stimulation. Indeed, phosphorothioate CpG oligonucleotides stimulated B cells at concentrations more than 2 logs lower than those required with unmodified oligonucleotides (Fig. 1c). Mice injected intraperitoneally with phosphorothioate CpG oligonucleotides had significant three- to sixfold increases in spleen B-cell proliferation and immunoglobulin secretion within 24 h ($P < 0.01$, not shown) and increased expression of activation markers such as class II major histocompatibility complex (MHC; Fig. 1d).

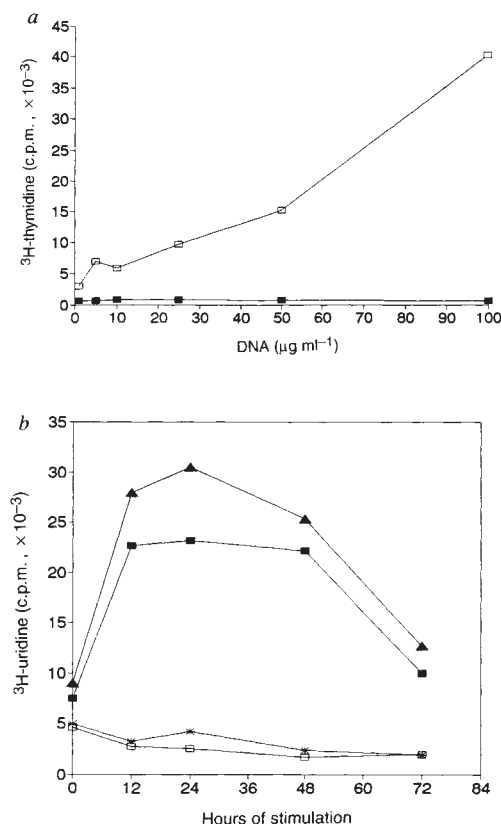
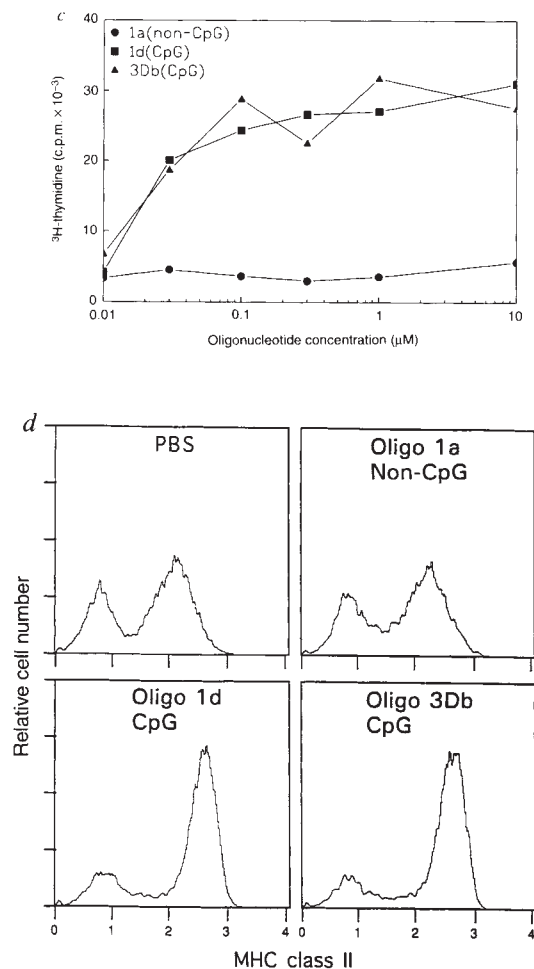


FIG. 1 a, B-cell stimulation by bacterial DNA requires unmethylated CpG dinucleotides. DBA/2 B cells (prepared as described in Table 1) were cultured with the indicated concentrations of heat-denatured (single-stranded) *E. coli* genomic DNA (which was 10–30% more mitogenic than dsDNA) for 44 h, then pulsed with ^3H -thymidine for 4 h before cell collection. The s.d. of the samples was $<10\%$. Where indicated, *E. coli* DNA was methylated with 2 U CpG methylase (New England Biolabs) per μg DNA for 18 h at 37°C (filled squares). Methylated DNA was completely protected against digestion with *Hpa*II but not *Msp*I. Bacterial DNA digested with DNase I for 30 min at 37°C was non-mitogenic. Unmethylated *E. coli* DNA (open squares) contained 2.5 ng of LPS per μg , and methylated DNA contained 12.5 ng LPS per μg by Limulus assay; both of which are below the LPS concentration which causes B-cell stimulation in our assay. Spleen B cells from C3H/HeJ mice cultured with unmodified *E. coli* DNA showed a similar dose response. As reported by Messina *et al.*² genomic DNA from NFS or NZB/N mouse liver caused minimal stimulation at any concentration (not shown). Unmethylated *E. coli* DNA induced an 8.8-fold increase in the number of IgM-secreting B cells by 48 h using the ELISPOT assay described in Table 1. b, Time course of oligonucleotide stimulation of ^3H -uridine uptake. DBA/2 spleen B cells were cultured with either no oligonucleotide (open squares), or the CpG oligonucleotides 1 (filled triangles) and 2 (filled squares) or control oligonucleotide 1a (star) as described in Table 1 except that the cells were pulsed with ^3H -uridine at the time indicated on the x-axis and collected 4 h later. c, Dose response of oligonucleotides for B-cell stimulation. DBA/2 spleen B cells were

Antigen-specific B-cell activation requires both antigen binding to surface immunoglobulin and one or more costimulatory signals. To determine whether CpG oligonucleotides could provide costimulatory signals, primary B cells were cultured with or without CpG oligonucleotide and/or a suboptimal dose of α -IgM, which activates B cells by crosslinking surface immunoglobulin. B cells exposed to both CpG oligonucleotide and α -IgM showed marked synergistic proliferation (Fig. 2a). CpG oligonucleotides also synergistically activated antigen-specific immunoglobulin secretion in the mouse B-cell line CH12.LX, the antigen receptor of which binds sheep red blood cells (SRBC) (Fig. 2b). These data indicate that the CpG motif preferentially



cultured as described in Table 1 with phosphorothioate-modified oligonucleotides with the CpG sequences 1d (squares) or 3Db (triangles) or the control sequence 1a (circles). CpG oligonucleotides with nuclease-resistant methylphosphonate internucleotide linkages induced less mitogenic effect (not shown). d, *In vivo* immune activation by CpG oligonucleotides. Mice were injected i.p. with 0.25 ml of sterile PBS or the indicated phosphorothioate oligonucleotide (sequences as shown in Table 1) dissolved in PBS at a dose of 500 μg per mouse, which has been reported to give levels in spleen tissue of $\sim 10 \mu\text{g g}^{-1}$ ($1.54 \mu\text{M}$)²⁸. Doses below 100 μg per mouse gave minimal stimulation. Twenty-four hours later, spleen cells were collected, washed and stained for flow cytometry using anti-class II MHC (M5/114). The peak on the left represents spleen T cells, which did not change in number, but became a smaller percentage of the total population because of B-cell proliferation. There were similar increases in expression of the B-cell activation markers Ly-6A/E, B7-2, Bla-1 and IgM (not shown). Two mice were studied for each condition and analysed individually. The experiment is one of four using several different mitogenic CpG oligonucleotides and six non-CpG oligonucleotides, which were all non-mitogenic.

activates B cells that simultaneously encounter their specific antigen.

The molecular mechanism by which CpG motifs induce B-cell activation is yet unclear. B cells do not appear to have a CpG-specific membrane receptor because there is no difference in their

TABLE 1 B-cell stimulation by CpG oligonucleotides

Oligonucleotide	Sequence*	Stimulation index	
		³ H Uridine†	IgM production‡
1	GCTAGACGTTAGCGT	6.1 ± 0.8	17.9 ± 3.6
1a	T.....	1.2 ± 0.2	1.7 ± 0.5
1b	Z.....	1.2 ± 0.1	1.8 ± 0.0
1c	Z.....	10.3 ± 4.4	9.5 ± 1.8
1d	..AT.....GAGC.	13.0 ± 2.3	18.3 ± 7.5
2	TCAACGTT	6.1 ± 1.4	19.2 ± 5.2
2a	GC..	1.1 ± 0.2	1.5 ± 1.1
2b	...G...C.	4.5 ± 0.2	9.6 ± 3.4
2c	...T...A.	2.7 ± 1.0	ND
2d	..TT...AA	1.3 ± 0.2	ND
2e		1.3 ± 0.2	1.1 ± 0.5
3D	GAGAACGCTGGACCTTCCAT	4.9 ± 0.5	19.9 ± 3.6
3Da	C.....	6.6 ± 1.5	33.9 ± 6.8
3Db	C.....G..	10.1 ± 2.8	25.4 ± 0.8
3Dc	...C.A.....	1.0 ± 0.1	1.2 ± 0.5
3Dd	Z.....	1.2 ± 0.2	1.0 ± 0.4
3De	Z.....	4.4 ± 1.2	18.8 ± 4.4
3Df	A.....	1.6 ± 0.1	7.7 ± 0.4
3Dg	CC.G.ACTG..	6.1 ± 1.5	18.6 ± 1.5
3M	TCCATGTTCGGTCCTGATGCT	4.1 ± 0.2	23.2 ± 4.9
3Ma	CT.....	0.9 ± 0.1	1.8 ± 0.5
3Mb	Z.....	1.3 ± 0.3	1.5 ± 0.6
3Mc	Z.....	5.4 ± 1.5	8.5 ± 2.6
3Md	A...T.....	17.2 ± 9.4	ND
3Me	C...A.....	3.6 ± 0.2	14.2 ± 5.2
LPS (30 µg ml ⁻¹)		7.8 ± 2.5	4.8 ± 1.0

The oligonucleotides shown are representative of more than 300 studied including: two previously reported as 'antisense' (oligonucleotides 3D and 3M; ref. 22); 48 8-base oligonucleotides; and 14 shorter oligonucleotides. CpG dinucleotides are underlined. Dots indicate identity, Z indicates 5-methylcytosine (Glen Research, Sterling, VA). Essentially identical results were obtained with oligonucleotides purchased from Operon Technologies (Alameda, CA) and from the DNA core facility, University of Iowa.

† Effects of oligonucleotides on B-cell RNA synthesis (as a measure of cell activation) were assessed using spleen cells from 6–12-week-old specific pathogen-free DBA/2 or BXSb mice that were depleted of T cells with anti-Thy-1.2 and complement and centrifugation over lympholyte M (Cedarlane Laboratories, Hornby, Ontario, Canada) as described²². Stimulation assays with 1 µCi of ³H uridine were done in 96-well microtitre plates with 20 µM oligonucleotide for 20 h. Similar stimulation was seen using C3H/HeJ B cells, demonstrating that the mitogenic effects were not due to LPS contamination, and using SJL/J B cells, which are hyporesponders to the 8-substituted guanosine B-cell mitogens. ³H-thymidine incorporation assays showed similar results, but with some nonspecific inhibition by thymidine released from degraded oligonucleotide²³. CpG-oligonucleotides did not induce detectable tyrosine phosphorylation of B-cell proteins, inositol trisphosphate generation, or Ca²⁺ mobilization within 10 min. Oligonucleotides caused no detectable proliferation of γδ or other T-cell populations.

‡ Resting B cells (<0.02% T-cell contamination) were isolated from spleen cells treated with anti-Thy1, anti-CD4, anti-CD8 and complement²⁴ from the 63–70% band of a discontinuous Percoll gradient²⁵. These were cultured as described above in 30 µM oligonucleotide or 20 µg ml⁻¹ LPS for 48 h. The number of IgM-secreting B cells was determined by ELISPOT assay²⁶. The background number of spots in unstimulated wells was 50 × 10⁶ to 60 × 10⁶ B cells. All assays were done in triplicate at least three times.

ND, Not done.

binding of fluorescent-tagged CpG and non-CpG oligonucleotides. CpG oligonucleotides linked to a solid support are non-stimulatory, indicating that cell uptake is required for activation (not shown). Double-stranded DNA is taken up by lymphocytes into an endosomal compartment where it is acidified and degraded to oligonucleotides over several hours⁴. Single-stranded oligonucleotides also appear to be taken up by B cells into an endosomal compartment⁵, indicating that both double-stranded DNA and single-stranded oligonucleotides may have the same intracellular locations and forms.

These results demonstrate a new T-cell-independent pathway of B-cell activation triggered by a CpG motif common in microbial but not vertebrate DNA. By providing a costimulatory signal to B cells that bind antigen, microbial DNA may aid the emergence of specific antimicrobial immune responses. This ability of the vertebrate immune system to be specifically activated by microbial DNA may confer a selective advantage by improving the host response to infection. These data also suggest a possible pathogenic role for the increased levels of hypomethylated CpG sequences reported in patients with the autoimmune disease systemic lupus erythematosus^{6,9}.

The origin of CpG suppression is unclear¹⁰. We propose that CpG suppression and/or methylation may have been selected for during vertebrate evolution so that immune defences might

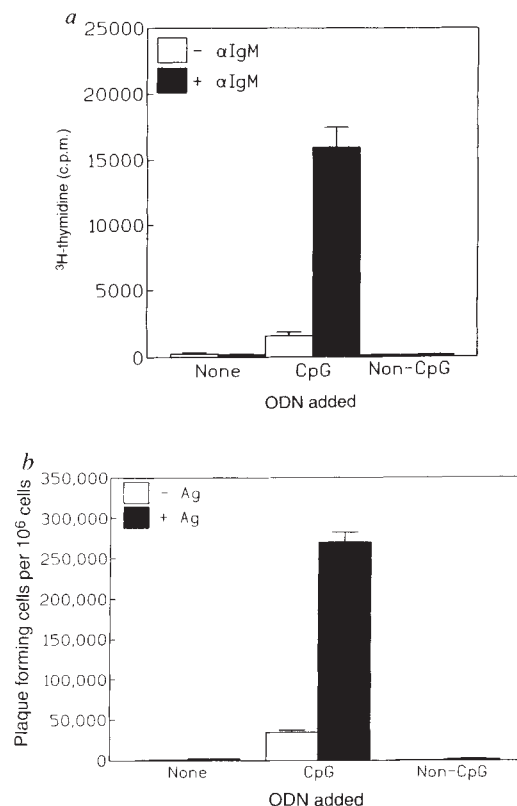


FIG. 2 Synergistic B-cell activation by CpG oligonucleotide and anti-IgM or antigen. a, Spleen B cells were prepared as in Table 1 and cultured with a monoclonal anti-IgM Ab (B-7-6, 1 µg ml⁻¹) and/or phosphorothioate CpG oligonucleotide 1d or control oligonucleotide 1a at 0.1 µM and ³H-thymidine incorporation measured after 48 h. The experiment was done three times with similar results. b, CH12.LX cells were cultured as previously described²⁹ for 72 h with or without antigen (0.1% SRBC) and/or 1 µg ml⁻¹ (~0.2 µM) of phosphorothioate-modified oligonucleotide 1d (CpG) or oligonucleotide 1a (Non-CpG). CH12.LX cells are not induced to secrete immunoglobulin by SRBC alone. IgM-secreting cells were enumerated as direct plaque-forming cells in a lawn of SRBC, as previously described²⁹. Plaque-forming cells are measured per 10⁶ viable recovered cells. Results are mean ± s.e.m. of duplicate cultures, and are representative of three experiments.

TABLE 2 Cell-cycle analysis with CpG oligonucleotides

Treatment	Percentage of cells in:		
	G0	G1	S + G2 + M
Media	97.6	2.4	0.02
Oligonucleotide 1a (non-CpG)	95.2	4.8	0.04
Oligonucleotide 1d (CpG)	2.7	74.4	22.9
Oligonucleotide 3Db (CpG)	3.5	76.4	20.1
LPS (30 µg ml ⁻¹)	17.3	70.5	12.2

B cells (2×10^6) were cultured for 48 h in 2 ml tissue culture medium alone, or with 30 µg ml⁻¹ LPS or with the indicated nuclease-resistant phosphorothioate oligonucleotide sequence at 1 µM. Cell-cycle analysis was as described²⁷. The results shown are representative of three experiments using several different CpG and non-CpG oligonucleotides.

be triggered preferentially by microbial DNA. This hypothesis predicts that mitogenic CpGs may be more suppressed than non-stimulatory CpGs. Indeed, among human coding DNA sequences in GenBank, the frequency of the four most stimulatory hexamers (GACGTC, GACGTT, AACGTC and AACGTT) is $0.72 \pm 0.13 \times 10^{-4}$, less than one-third that of the four predicted most unfavourable CpG-containing sequences, (TCCGGA, TCCGGG, CCCGGA and CCCGGG), which was $2.22 \pm 0.94 \times 10^{-4}$ (ref. 11). In contrast, the frequencies of these hexamers in *Escherichia coli* DNA are $2.67 \pm 0.86 \times 10^{-4}$ and $2.17 \pm 0.87 \times 10^{-4}$, respectively (J. Han, personal communication), not significantly different from that of the non-mitogenic hexamers in humans.

Antisense oligonucleotides targeted to complementary messenger RNAs are popular research tools¹²⁻¹⁵ and are now in human clinical trials¹⁶⁻¹⁹. It has become increasingly clear that oligonucleotides have many non-antisense effects^{15,20,21}. Our review of the literature reveals 18 reports in which oligonucleotides that cause *in vitro* or *in vivo* lymphocyte activation or protection from apoptosis contain excellent matches for the CpG motif whereas control oligonucleotides do not (list available from authors on request). It may be desirable to avoid unmethylated CpGs in 'antisense' oligonucleotides, or at least to include controls for these effects. By contrast, immune-stimulating CpG oligonucleotides may prove clinically useful as adjuvants and biological response modifiers. □

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Anti-inflammatory properties of a platelet-activating factor acetylhydrolase

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PLATELET-ACTIVATING factor (PAF) is a potent pro-inflammatory phospholipid that activates cells involved in inflammation^{1,2}. The biological activity of PAF depends on its structural features, namely an ether linkage at the *sn*-1 position and an acetate group at the *sn*-2 position. The actions of PAF are abolished by hydrolysis of the acetyl residue, a reaction catalysed by PAF acetylhydrolase. There are at least two forms of this enzyme—one intracellular and another that circulates in plasma and is likely to regulate inflammation. Here we report the molecular cloning and characterization of the human plasma PAF acetylhydrolase. The unique sequence contains a Gly-Xaa-Ser-Xaa-Gly motif commonly found in lipases. Recombinant PAF acetylhydrolase has the substrate specificity and lipoprotein association of the native enzyme, and blocks inflammation *in vivo*: it markedly decreases vascular leakage in pleurisy and paw oedema, suggesting that PAF acetylhydrolase might be a useful therapy for severe acute inflammation.

We purified PAF acetylhydrolase from human plasma (Fig. 1 legend). As macrophages secrete PAF acetylhydrolase activity *in vitro*^{3,4}, we made a plasmid complementary DNA library from messenger RNA from monocyte-derived macrophages⁵, which we screened using a combination of the polymerase chain reaction (PCR) and hybridization for the unique N-terminal sequence (IQVLMASFGQTKIP) of the purified protein (*M*_r 44K). A full-length clone (sAH406-3) contains a 1.5-kilobase (kb) insert that encodes a predicted 441-amino-acid protein (Fig. 1). The open reading frame is defined by a translation initiation codon at nucleotide 160 that is preceded by a stop codon 24 base pairs (bp) upstream. The translation termination codon at the 3' end lies immediately upstream of the predicted polyadenylation sequence (ATTAA). Interestingly, the amino terminus determined by protein sequencing lies 42 residues downstream of the predicted initiating methionine. Hydropathy analysis suggests a hydrophobic signal peptide within the first 20 residues and, consistent with this, Ala 17/Val 18 constitutes a reasonable consensus signal cleavage position⁶. Lys 41/Ile 42 is unlikely to

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represent the signal cleavage site, because lysine has not been found at the -1 position⁶. The remaining peptide from residues Val 18 to Lys 41 may be either a propeptide that is cleaved by post-translational modification or a fragment of the mature protein that was cleaved during purification. The predicted size of residues 42-441 is $M_r = 45,388$ which compares favourably with the observed size of the natural enzyme on SDS-PAGE. The predicted protein sequence was compared with the Genbank database and found to be unique, apart from a Gly-Xaa-Ser-Xaa-Gly (GXSG) motif (at Ser 273) characteristic of lipases and esterases^{7,8}. The serine in this motif is the active-site nucleophile in these families. The presence of this sequence in plasma PAF acetylhydrolase is consistent with its role as a phospholipase, and explains the inhibition by the serine esterase inhibitor diisopropylfluorophosphate (DFP)⁹.

To determine definitively whether cDNA sAH406-3 encodes PAF acetylhydrolase activity, it was subcloned into both mammalian and *Escherichia coli* expression vectors. For mammalian cell expression, we transiently transfected COS cells with full-length cDNA, and found that they secreted $5 \text{ nmol ml}^{-1} \text{ h}^{-1}$ of activity into the medium (control supernatants contained $0.4 \text{ nmol ml}^{-1} \text{ h}^{-1}$). For *E. coli* expression, the coding region of sAH406-3 beginning with Ile 42 was inserted with the *trp* promoter and a translational start codon into the multiple cloning site of pUC19. Homogenates of the *E. coli* transformants had an average of $57.5 \mu\text{mol ml}^{-1} \text{ h}^{-1}$ of PAF acetylhydrolase activity; none was detected in mock transformants.

FIG. 1 Nucleotide and deduced amino-acid sequences of a cDNA encoding human plasma PAF acetylhydrolase. Amino acids are numbered beginning with the presumed initiating methionine. Isoleucine 42, the N-terminal amino acid of purified plasma PAF acetylhydrolase, is doubly underlined; the conserved lipase active-site serine motif is boxed; and the putative polyadenylation hexanucleotide is underlined. The Genbank accession number is U20157.

METHODS. PAF acetylhydrolase was purified from human low-density lipoprotein (LDL) particles prepared as described¹¹. LDL particles solubilized in 25 mM Tris, pH 7.5, containing 10 mM CHAPS were chromatographed on DEAE-Sephacrose Fast Flow followed by Blue Sepharose Fast Flow and Cu^{2+} -charged chelating Sepharose (all from Pharmacia). This yielded a purification >200,000-fold. For a further 10-fold purification, a portion of the preparation was separated by SDS-PAGE, and activity was found in gel slices at M_r 44K¹¹. Accordingly, another aliquot was reduced in 50 mM DTT, electrophoresed through a 7.5% polyacrylamide gel containing 0.1% SDS, electroblotted to a polyvinylidene fluoride membrane, and stained with 0.1% Coomassie blue. A protein band of M_r 44K, with which PAF acetylhydrolase activity comigrated, was excised from the membrane and the N-terminal sequence was determined by automated Edman degradation. PAF acetylhydrolase activity was determined as described⁹. A degenerate oligonucleotide PCR primer (5'-ACATGAATTCGGIATC/TTTIGTC/TTGICC-A/GAA-3', where I is inosine) was used to screen a monocyte-derived macrophage cDNA library. The library was synthesized with the Copy Kit (Invitrogen) in a plasmid vector, pRc/CMV, which contains the SP6 and T7 promoter sequences flanking the cloning site. Using the degenerate PCR primer in combination with primers specific for either of the promoter regions, a fragment containing nucleotide sequence consistent with the N-terminal sequence of the purified protein was isolated. New PCR primers (sense: 5'-TATTCTAGAAAGTGGTGGAACTCGCTGG-3'; antisense: 5'-CGATG-AATTCAGCTTGCAGCAGCCATCAGTAC-3') based on this fragment were synthesized and used to identify a pool of 3,000 clones containing a

The biochemical property that defines PAF acetylhydrolase is its specificity for substrates with a short residue at the *sn*-2 position^{10,11}. The enzyme is inactive against long-chain phospholipids, a property that explains how it can circulate in an active form without resulting in hydrolysis of lipids in blood cell membranes and lipoproteins. However, it catalyses the hydrolysis of PAF and of structurally similar phospholipids resulting from oxidative fragmentation of the *sn*-2 fatty acid¹². Our initial assays confirmed that the recombinant enzyme recognized PAF. To determine whether it has the specificity of the plasma enzyme, a phospholipid with a long-chain fatty acid in the *sn*-2' position, 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (arachidonoylPC), was tested and found not to be a substrate (Fig. 2a). But a phospholipid with a short chain, 1-palmitoyl-2-glutaroyl-*sn*-glycero-3-phosphocholine (glutaroylPC), which is derived from arachidonoylPC by oxidation of the arachidonoyl group, was hydrolysed when incubated with PAF acetylhydrolase (Fig. 2a). Thus, recombinant PAF acetylhydrolase exhibits the same substrate selectivity as the native enzyme. Additional distinctive properties of plasma PAF acetylhydrolase include calcium-independence and resistance to compounds that modify sulphydryl groups or disrupt disulphides¹¹. However, it is sensitive to DFP, indicating that a serine comprises part of the active site. The recombinant enzyme behaved identically: it was impervious to treatment with EDTA, DTT, and 5,5'-dithiobis-(2-nitrobenzoic acid), but was inhibited by 2 mM DFP (in three experiments). Like the native activity, the recombinant enzyme associates with

TGGTCCGGAGGCTCGCAGTCTGTCTGGCGAGAAGCAGTCCGGTTGGAGCGCTGGCTCGCGTGTGTCGCGGTGGAACGCGCCAGGGAC	90
CCCAGTTCGCCGAGCAGCTCCGCGCGCGCTGAGAGACTAAGCTGAACTGCTGCTCAGTCCCAAGATGGTGCACCCCAATTCGAT	180
M V P P K L H	7
GTGCTTTTCTGCCTCTCGCGCTGCCTGGCTGTGGTTTATCTTTTACTGGCAATACATAAATCTGTGCCCATATGAATCATCAGCA	270
V L F C L C G C L A V V Y P F D W Q Y I N P V A H M K S S A	37
TGGGTCAACAAATACAAGTACTGATGGCTGCTGCAAGCTTTGGCCAACTAAATCCCGCGGGAATGGGCTTATTCGCTGGTGT	360
W V N K I Q V L M A A S F G Q T K I P R G N G P Y S V G C	67
ACAGACTTAATGTTTGTACACACTAATAAGGGCACTTCTTGGCTTTATATTATCCATCCAGATATATGATCGCTTACACCCCTTGG	450
T D L M F D H T N K G T F L R L Y Y P S Q D N D R L D T L W	97
ATCCCAATTAAGAAATATTTTGGGGTCTTATGCAAAATTTCTTGGAAACACTGGCTTATGGGCAACATTTTGGGTACTCTTGGTTC	540
I P N K E Y F W G L S K F L G T H W L M G N I L R L L F G S	127
ATGCAACTCTCTGCAAACTGGAATTCCTCTGAGGCTGGTGAAATAATCCACTTGTGTTTTTCTCATGTCTTGGGGCATTTCAGG	630
M T T P A N W N S P L R P G E K Y P L V V F S H G L G A F R	157
ACACTTTATCTGCTATTGGCATTGACCTGGCATCTCATGGGTTTATAGTTGTCTGTAGAACACAGAGATAGATCTGCATCTGCAACT	720
T L Y S A I G I D L A S H G F I V A A V E H R D R S A S A T	187
TACTATTTCAAGGCAATCTGCTGCAGAAATAGGGCAAGCTTGGCTCTACCTTAGAACCTTGAACCAAGAGGAGGAGACATATA	810
Y Y F L K D Q S A A E I G D K S W L Y L R T L K Q E E T H I	217
CGAAATGACGAGTACGCGCAAGAGCAAGAAATGTTCCCAAGCTCTCAGTCTGATTCTTGACATTGATCATGGAAGCCAGTGAAGAAT	900
R N E Q V R Q R A K E C S Q A L S L I L D I D H G K P V K N	247
GCATTAGATTTAAAGTTTGATAGGAACCACTGAAGGACTCTATTGATAGGGAATAATAGCAATTTGGCAATCTTTTGGTGGAGCA	990
A L D L K F D M E Q L K D S I D R E K A I V I G H S F G G A	277
ACGGTTATTGACACTCTTAGTGAAGATCAGAGATTCAGATGTGGTATTGCCCTGGATGCATGGATGTTTCCACTGGGTGATGAAGTATAT	1080
T V I Q T L S E D Q R F R C G I A L D A W M F P L G D E V Y	307
TCCAGAACTCTCAGCCCTCTTTTATCACTCTGAATATTTCCAAATCTCTGCTAATATCATAAAATGAAATATGCTACTCACCT	1170
S R I P Q P L F F I N S E Y F Q Y P A N I I K M K K C Y S P	337
GATAAAGAAAGAAATGATGATACAACTCAGGGGTCAGTCCACCAGAAATTTGTGACTTCACTTTTGCAGTGGCAAAATATTTGGACAC	1260
D K E R K M I T I R G S V H Q N F A D F T F A T G K I I G H	367
ATGCTCAAAATTAAGGGAGACATAGATCAAAATGTAGCTATTGATCTTAGCAACAAGCTTCATTAGCATCTTACAAAGCATTTAGGA	1350
M L K L K G D I D S N V A I D L S N K A S L A F L Q K H L G	397
CTTCATAAAGATTTGATCAGTGGGACTGCTGATTGAAGGAGATGAGAATCTTATTCAGGGACCAACATTAACACCAACCAATCAA	1440
L H K D F D Q W D C L I E G D D E N L I P G T N I N T T N Q	427
CACATCATGTTACAGAACTCTTCCAGGAATAGAGAAATACAAATAGGATTAAGATAGGTTTTTAAAAA	1518
H I M L Q N S S G I E K Y N	441

PAF acetylhydrolase clone. The pool was screened by hybridization using the PCR fragment labelled with ³²P by hexamer random priming as a probe. Colonies lifted onto nitrocellulose filters were prehybridized and hybridized in Denhardt's solution containing 50 $\mu\text{g ml}^{-1}$ sonicated salmon sperm DNA and 50% formamide. After overnight hybridization at 42 °C, blots were washed in 0.2 × SSC and 0.1% SDS. The nucleotide sequence of hybridizing clones was determined by the dideoxy chain-termination method (Sequenase; US Biochemical).

low- and high-density lipoproteins⁹ (Fig. 2b). These results confirm that the cDNA sAH406-3 encodes plasma PAF acetylhydrolase.

The source of PAF acetylhydrolase found in the plasma is not known, but cultured macrophages secrete large amounts of enzyme^{3,4}, and hepatocytes secrete less^{13,14}. Northern blot analysis of human tissues using radiolabelled cDNA revealed a 1.8-kb band in thymus, tonsil and, to a lesser extent, placental RNA. There was no hybridization to RNA from heart, kidney, liver or cerebral cortex (Fig. 3a). We have observed expression in other regions of the brain (manuscript in preparation). The lack of PAF acetylhydrolase mRNA expression in the liver was surprising but reproducible and was confirmed by *in situ* hybrid-

ization. These results implicate macrophages as the likely source of plasma PAF acetylhydrolase *in vivo* as the positive tissues have large numbers of macrophages. In previous studies, monocytes were found not to secrete enzyme activity until they differentiated into macrophages^{3,4}. We confirmed this result (Fig. 3b) and showed that there was no mRNA for PAF acetylhydrolase in freshly isolated monocytes, but expression was induced and maintained during differentiation into macrophages.

PAF provokes inflammation in part by its ability to activate polymorphonuclear neutrophils (PMNs), which results in an increase in intracellular calcium, secretion of granule products, spreading on surfaces and polarization, which is required for directional migration¹⁵. We found that PAF preincubated with recombinant PAF acetylhydrolase lost its ability to activate polarization and spreading *in vitro* (Fig. 4a). Increased vascular permeability is a prominent component of severe inflammation, and PAF is a potent stimulus for this response. Using the well characterized paw oedema model¹⁶, we found that injection of PAF between the hind footpads of rats reproducibly induced oedema. Pretreatment of the footpads with recombinant PAF acetylhydrolase blocked PAF-induced oedema by >85%. In a representative experiment, the foot volume increased by 0.63 ml

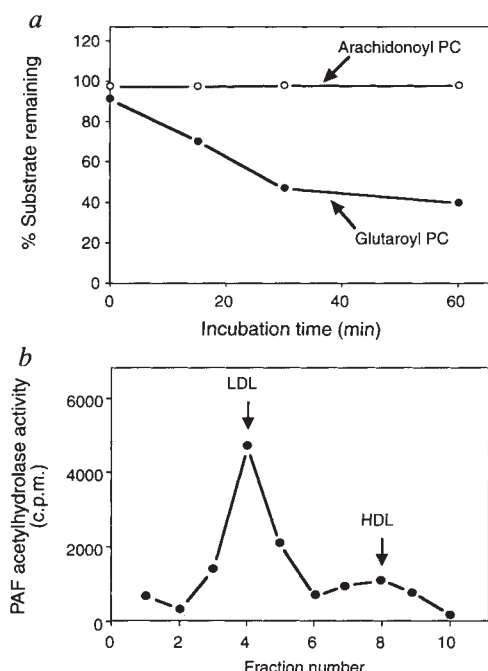


FIG. 2 Recombinant PAF acetylhydrolase hydrolyses an oxidized, but not native, phospholipid (a) and associates with plasma lipoproteins (b). a, To determine substrate specificity, recombinant PAF acetylhydrolase was incubated with either 1-[¹⁴C]palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine ('arachidonoyl PC'), or with 1-[¹⁴C]palmitoyl-2-glutaryl-sn-glycero-3-phosphocholine ('Glutaryl PC'). The rate of hydrolysis of each phospholipid, which differed only at the acyl residue at the sn-2 position, was determined by extracting the lipids from the reaction mixture at the indicated times and determining the amount of substrate remaining (and lysophospholipid accumulated) after separation by thin-layer chromatography¹². This experiment was done twice, with equivalent results each time. b, The plasma lipoprotein association of recombinant PAF acetylhydrolase was determined by incubating the enzyme with 10% human serum that had previously been treated with DFP to inactivate the native PAF acetylhydrolase. The incubation was carried out at pH 7.4, and then the lipoprotein fractions were isolated by centrifugation in a density gradient and assayed for PAF acetylhydrolase activity. This experiment was performed three times with the same result each time.

METHODS. The expression system consisted of a pUC19 vector containing the Trp promoter²⁷ immediately upstream of a partial PAF acetylhydrolase cDNA generated by PCR. The 5' sense PCR primer (5'-TATTCTAGAAATTATGATACAAGTATTATGCTGCTGCAAG) contained a translation initiation codon followed by eight codons for the mature PAF acetylhydrolase beginning at Ile 42. The 3' antisense primer (5'-ATTGATATCCTAATTGTATTCTCTATTCTCTG) encompassed the termination codon of the PAF acetylhydrolase cDNA. *E. coli* strain XL-1 was transformed with the vector, cultured in L-broth containing 0.1 mg ml⁻¹ carbenicillin, and lysed in 25 mM Tris containing 10 mM CHAPS. The enzyme was purified by chromatography on S-Sepharose Fast Flow and Blue Sepharose Fast Flow (Pharmacia). The recombinant enzyme ran as a single 44K band on SDS-PAGE and was ~95% pure.

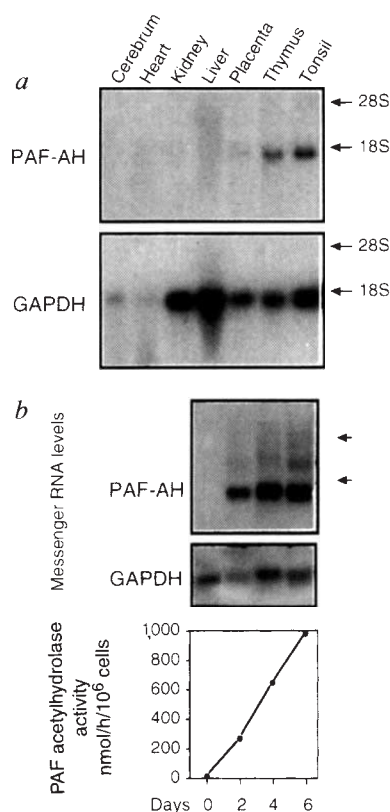
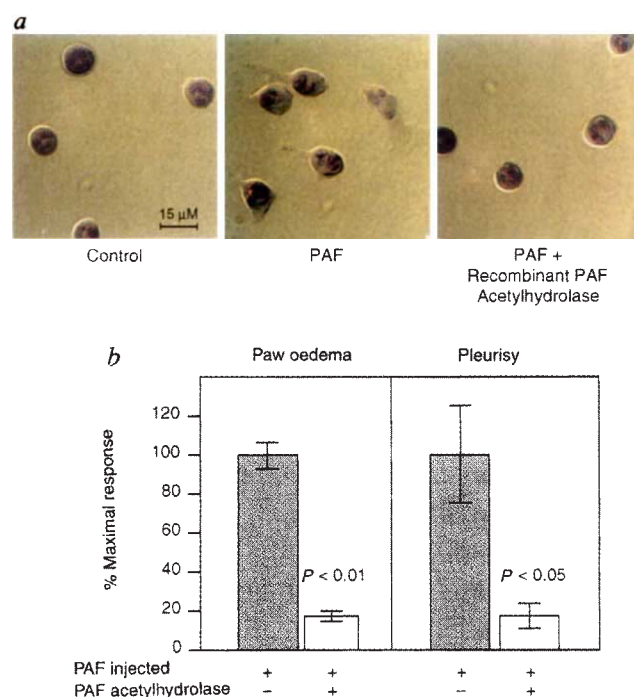


FIG. 3 Analysis of PAF acetylhydrolase expression in human tissues and monocyte-derived macrophages. a, Total cellular RNA was prepared from the indicated human tissues obtained from Cooperative Health Tissue Network (Cleveland, OH). The RNA was electrophoresed, transferred to nitrocellulose blots, and hybridized first with a radioactively labelled PAF acetylhydrolase (PAF-AH) cDNA probe, then with a labelled glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe. The positions of the 18S and 28S ribosomal bands are indicated. The results are from a single experiment representative of three. b, Monocytes isolated from human blood were cultured for 0 to 6 days, during which time they developed characteristics of macrophages. At each time indicated, total cellular RNA was extracted and northern blots prepared. Arrows denote the positions of the 18S and 28S ribosomal bands. In addition, culture medium from each developmental time point was assayed for PAF acetylhydrolase activity. The results are from a single experiment.

FIG. 4 Recombinant PAF acetylhydrolase protects against PAF-mediated inflammation *in vitro* and *in vivo*. **a**, Purified recombinant PAF acetylhydrolase was incubated with 10^{-7} M PAF for 15 min (37 °C). The controls contained either PAF or the enzyme alone. After incubation, portions were removed and added to isolated human neutrophils⁵ (final concentration of PAF was 10^{-8} M in the samples without PAF acetylhydrolase). After 30 min, the cells were stained with Diff-Quik and viewed under Nomarski optics. **b**, Rats were pretreated intravenously with recombinant PAF acetylhydrolase (open bars) or with carrier (filled bars). Fifteen minutes later, animals were challenged with PAF. In the paw oedema test, the hind footpad was injected with PAF and oedema was assessed 1 hour later. In the pleurisy test, PAF was injected into the pleural cavity and after 1 h the exudate was quantified. All results are the mean $\pm$ s.e.m. % injury from four animals per group in one experiment. The same results, in both models, were obtained in multiple experiments carried out with slightly different protocols.

METHODS. Both models have been described¹⁶. PAF was suspended in 150 mM NaCl, 10 mM Tris, pH 7.5, and 0.25% BSA by sonication for five minutes. For the footpad test, female Long Evans rats (Charles River, 6–8 weeks old, mass 180–200 g) received 50 μ l (1.0 nmol) subcutaneously between the hind footpads. Oedema was quantified by measuring the foot volume (in ml) immediately before administration of PAF and again 1 h post-challenge using a plethysmometer (UGO Basile). Before PAF challenge, rats received 300 μ l of recombinant PAF acetylhydrolase (1500 μ mol ml⁻¹ h⁻¹) or carrier in the tail vein. In the pleurisy test, rats were injected intrapleurally with 100 μ l (2.0 nmol) PAF. Before challenge, rats were injected in the tail vein with 200 μ l of 1% Evans blue dye in 0.9% saline with either 300 μ l PAF acetylhydrolase (1500 μ mol ml⁻¹ h⁻¹) or carrier. Rats were killed after 1 h and pleural fluid was collected by rinsing the cavity with 3 ml heparinized phosphate-buffered saline. Vascular leakage into the pleural space was quantified by absorbance at 620 nm, a measure of dye concentration. Results were normalized to plasma dye levels to account for injection efficiency and reported as percentage of the value in controls. The differences between treatment groups were analysed with a paired t-test.



(± 0.14 ml) for the PAF group (4 animals), but in the group that was treated with PAF acetylhydrolase before receiving PAF the increase was only 0.08 ml (± 0.02 ml). Similarly, intravenous pretreatment with recombinant PAF acetylhydrolase blocked PAF-induced footpad oedema by more than 80% (Fig. 4b).

We also tested PAF acetylhydrolase in a pleurisy model characterized by vascular leakage into the pleural space in response to PAF¹⁶. Rats were pretreated with either recombinant PAF acetylhydrolase or control buffer, then PAF was injected into the pleural space, and one hour later the degree of vascular leakage was determined. The rats that had received PAF acetylhydrolase had a >80% reduction of vascular leakage compared to control animals (Fig. 4b). Collectively, the *in vivo* and *in vitro* results demonstrate that PAF acetylhydrolase abolishes the inflammatory effects of PAF on leukocytes and the vasculature.

Human plasma PAF acetylhydrolase is a novel extracellular phospholipase A₂ that is physiologically and pathologically important because it abolishes the diverse, potent activities of PAF and oxidized phospholipids^{17–20}. The plasma PAF acetylhydrolase shares an active-site serine motif with the intracellular enzyme from bovine brain^{21,22}, but otherwise these proteins have no sequence similarity²¹. In addition, the cDNA described by Hattori *et al.* contains no signal sequence²¹, which is consistent with its intracellular location—unlike the enzyme described here, which is secreted. The tissue distribution of the intracellular enzyme has not been reported, but may provide clues as to whether the two forms have overlapping or distinct functions. Presumably the cytoplasmic form functions to regulate intracellular actions of PAF or PAF-like phospholipids, whereas the secreted enzyme can control the magnitude and duration of PAF-mediated signalling between cells. This predicts that a decreased ability to degrade PAF would result in pathological responses. This is supported by reports that the activity of

plasma PAF acetylhydrolase is decreased in patients with asthma²³, systemic lupus erythematosus²⁴ and septic shock²⁵. Moreover, one physiological action of PAF may be to initiate parturition, and an inappropriately low level of PAF acetylhydrolase may contribute to premature onset of labour²⁶. Our results show that the administration of supplemental, exogenous PAF acetylhydrolase suppresses inflammation. Thus, in diseases in which there is a deficiency of enzyme activity, PAF or related compounds might accumulate, and supplemental PAF acetylhydrolase may reverse the pathological response. □

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Elastic distortion of myosin heads and repriming of the working stroke in muscle

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MUSCLE contraction is driven by a cyclical interaction between the globular head domain of myosin and the actin filaments^{1–6}. We used quick stretches of 5 nm per half sarcomere to synchronize the movements of myosin heads in active single muscle fibres^{5,7,8}. The intensity of the 14.5 nm X-ray reflection decreased during the stretch, showing that the instantaneous elasticity of muscle^{1,5} involves distortion of myosin heads. Head movement continued at about $1,500\text{ s}^{-1}$ after the stretch, accompanied by partial force recovery. This indicates a reversal of the force-generating 'working stroke' in the myosin heads^{5,8,9} that is smaller and faster than assumed previously^{5,10,11}. By 50 ms after the stretch, myosin heads have returned both their original conformation and the ability to execute a normal working stroke. This 'repriming' process is slower than that following shortening¹² but much faster than the ATP turnover rate per myosin head.

Alternate stretching and shortening steps were imposed many times in each activation of single muscle fibres so that the X-ray signals could be summed. A stretch of 5 nm per half sarcomere was followed after 4 ms by a 5 nm shortening, then after 16 ms the cycle was repeated. With this protocol each stretch starts from the isometric force level, and each force transient is similar to that produced by a single stretch (Fig. 1a). The intensity of the 14.5 nm X-ray reflection from the axial repeat of the myosin heads along the filaments^{7,13} was measured at 100- μs intervals (Fig. 1b), ten times faster than in previous measurements on whole muscles⁷. The improved time resolution reveals a step decrease in intensity of about 25% at the stretch, followed by a slower decrease of similar extent over the next 2 ms. A decrease in the intensity of the 14.5 nm reflection indicates tilting of myosin heads towards the filament axis so their mass is more uniformly spread along it^{7,8}. The approximate extent of tilting may be estimated from a simple structural model⁸, and the

intensity decrease during a 5 nm stretch would require movement of the tip of the head by a few nanometres. Thus a significant part of the instantaneous elasticity of muscle, which is thought to be responsible for coupling structural changes to force production^{1,5}, appears to reside within the myosin head.

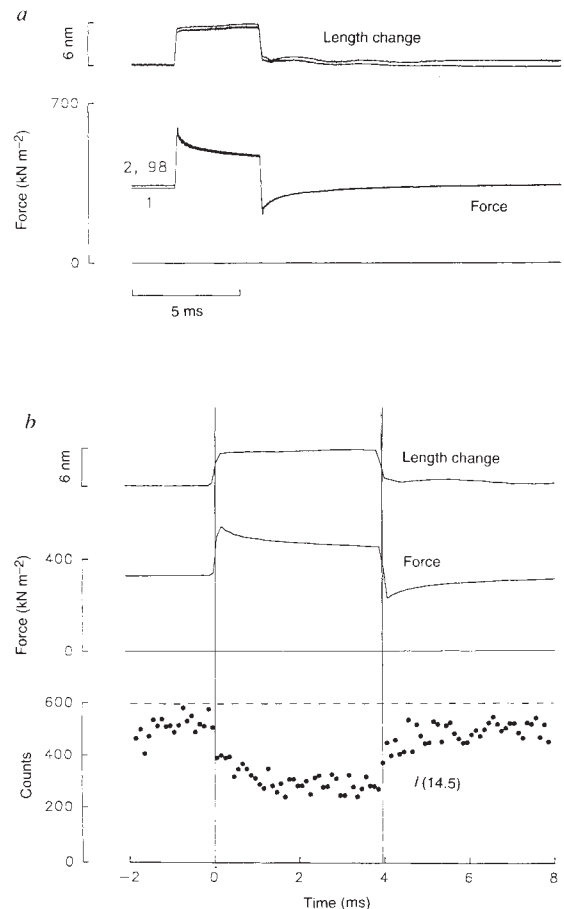
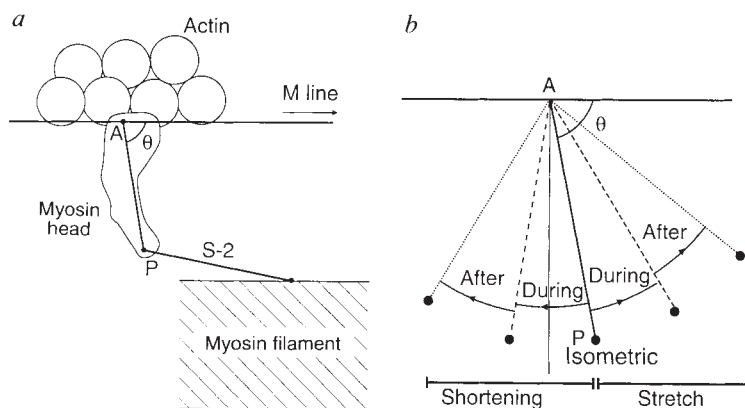


FIG. 1 Changes in force and the intensity of the 14.5 nm X-ray reflection ($I(14.5)$) produced by a 5 nm stretch followed 4 ms later by a 5 nm shortening step, with the cycle repeated every 20 ms. **a**, Superimposed segment length change in nm per half sarcomere and force records during the first, second and 98th of a series of 100 cycles, starting after 0.25 s of a 2.3 s tetanus. Fibre cross-sectional area, $22,400\text{ }\mu\text{m}^2$; initial fibre length, 5.8 mm; initial mean sarcomere length in the 2.37 mm segment interrogated by the X-ray beam, $2.13\text{ }\mu\text{m}$. **b**, Segment length change in nm per half sarcomere and force from the 98th cycle in **a**, and $I(14.5)$ averaged from 100 cycles. Length and tension shown at 100 μs time resolution for comparison with $I(14.5)$; these 100- μs points were used for exponential fitting. X-ray data from 79 tetani in 7 fibres, including that in **a**. The broken horizontal line denotes $I(14.5)$ in tetani without imposed steps in the same fibres. The vertical lines denote the midpoints of the 0.12 ms length steps. There was no significant change in the width of the 14.5 nm reflection either parallel or perpendicular to the fibre axis, or in the intensities of the inner equatorial reflections¹⁴.

METHODS. Mechanical and X-ray measurements on intact single fibres from anterior tibialis muscles of *Rana temporaria* at 3–4 °C were made at the Synchrotron Radiation Source (Daresbury, UK) as described previously⁸. Details of the motor^{11,18}, force transducer¹⁹, striation follower²⁰, and X-ray camera²¹ have been published. The fibre-to-detector distance was 2.2 m. X-ray data analysis used the BSL and OTOKO packages from Daresbury Laboratory. The background under the X-ray reflections was determined by linear fitting and subtracted. The intensity of the 14.5 nm meridional reflection was calculated by integrating from reciprocal spacings of $1/13.4$ to $1/15.8\text{ nm}^{-1}$ axially and $1/180\text{ nm}^{-1}$ on either side of the meridian.

FIG. 2 Simplified model of changes in the orientation of myosin heads produced by a 5 nm length step from the isometric state. *a*, Schematic representation of a myosin head bound to the actin filament (drawn after ref. 6) and attached to the myosin filament via the subfragment-2 (S-2) region at P. We consider only a simple rotation of the whole myosin head about the actin attachment point A, changing the angle θ between AP and the filament axis. The intensity of the 14.5 nm X-ray reflection depends on the mass distribution of the myosin heads projected onto the filament axis, and therefore on θ . The detailed shape of the head and possible motions between domains are ignored, so the projected mass of the myosin heads exhibits the sharpest 14.5 nm periodicity along the filaments, giving the most intense 14.5 nm reflection, when $\theta = 90^\circ$. Any tilting of the heads away from this perpendicular conformation spreads the projected mass along the filaments and decreases the 14.5 nm reflection intensity. *b*, The orientations of the AP vector in the isometric state (thick line), at the end of a stretch or shortening step starting from the isometric state (broken lines), and following the subsequent rapid force recovery (dotted lines), all drawn relative to the actin attachment A. The transitions are labelled as occurring during or after the shortening step or stretch, and the orientation changes are exaggerated for clarity. For simplicity each



transition is assigned the same angle change, although the rotation after shortening steps⁸ may be larger than that after stretch (Fig. 1*b*). Following conventional cross-bridge models⁵, the myosin heads are allowed to rotate after a length step, in the working stroke or its reversal, because there is assumed to be another elasticity in series with them.

We showed previously that the intensity of the 14.5 nm reflection does not change during the elastic response to 6 nm shortening steps starting from near the isometric force level⁸. However, the intensity increases during a shortening step applied 4 ms after a stretch (Fig. 1*b*). The elastic signal is similar for stretches and shortening steps over the same force range, but is only seen if the force is above the isometric level. This nonlinearity is in marked contrast to the mechanical elasticity, which is close to linear from zero force up to at least four times the isometric force^{9,11}. The discrepancy could be explained if the long axis of the myosin head were offset from the perpendicular to the filament axis in the isometric state (Fig. 2). During a 6 nm shortening step from the isometric state the heads would move through the perpendicular, with no net effect on the distribution of mass along the filaments or the intensity of the 14.5 nm X-ray reflection. After shortening, the tilting of the myosin head associated with the working stroke would produce a delayed intensity decrease, as observed⁸. This model can also explain the absence of an intensity decrease after a shortening step of only 2 nm¹⁴.

The decrease in intensity of the 14.5 nm reflection caused by a stretch from the isometric state (Fig. 1*b*) can be explained by the heads tilting towards the filament axis both during and after the stretch (Fig. 2). Single-exponential fits to the intensity and force data in the 4 ms after a 5 nm stretch (Fig. 1*b*) gave rate constants of 1,470 s⁻¹ and 930 s⁻¹, respectively. These fits give only rough estimates of the rates of the early recovery components, as slower components also contribute in the 4 ms period. Nevertheless, the similarity of the rate constants suggests that the ~1,500 s⁻¹ component of the X-ray signal is due to reversal of the force-generating working stroke seen after shortening steps^{5,8}.

The change in intensity of the 14.5 nm X-ray reflection following a shortening step 4 ms after a stretch (Fig. 1*b*) is in the opposite direction to that following a shortening step from the isometric state⁸. Also the force recovery following a shortening step 4 ms after a stretch (thick line in Fig. 3) is smaller and slower than that following a shortening step applied in the isometric state (thin line). Such an effect is predicted by contraction models based on that of Huxley and Simmons^{5,15} if myosin heads remain under strain for at least 4 ms after stretch because they are still attached to the same actin monomer. This would also explain why the structural changes produced by a stretch can be reversed by imposing shortening after 4 ms (Fig. 1*b*). The modified working stroke elicited by such a shortening step would

involve heads tilting away from the filament axis as the isometric orientation is regained (Fig. 2).

If a longer interval is allowed after a stretch the normal response to a shortening step is regained (Fig. 4). Here a 5 nm shortening step was followed 20 ms later by a 5 nm stretch; after a further 48 ms the cycle was repeated. Each shortening step starts from near the isometric force level and elicits a normal rapid force-generating process (Fig. 4*a*). By 48 ms after a stretch, the memory of the stretch has been lost, suggesting that the myosin heads have detached from actin and reattached in a state capable of repeating the elementary force-generating process, analogous to the 'repriming' process previously described after shortening¹². The X-ray signals obtained by summing many cycles of this protocol (Fig. 4*b*) are consistent with this interpretation. Both shortening and stretch now produce a decrease in the intensity of the 14.5 nm reflection, even though each shortening step occurs after a stretch in the steady state. The intensity decrease following shortening accompanies the elementary force-generating process, and its recovery has a half-time of about 10 ms, matching that of repriming^{8,12}. A stretch imposed 20 ms after shortening produces a decrease in the X-ray intensity similar to that in Fig. 1*b*, followed by recovery with a half-time of 23 ms. Similar behaviour of the 14.5 nm reflection has been observed with 1 ms time resolution in whole muscles⁷.

The slow part of the force recovery after stretch, which has a half-time of about 20 ms (Fig. 4*b*), accompanies repriming, as

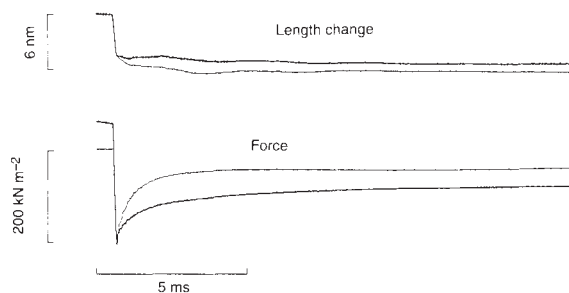


FIG. 3 Superimposed segment length in nm per half sarcomere and force changes from the shortening step in the second cycle of Fig. 1*a* (thick lines) compared with the response to a single shortening step of the same amplitude applied to the isometric fibre (thin lines). The fibre is stiffer after the stretch¹¹, as indicated by the larger force decrease during the shortening step, but the amplitude of the rapid force recovery is much smaller.

myosin heads detach from actin and the isometric conformation is regained. Thus the slow force recovery is probably due to detachment of strained myosin heads rather than to reversal of the working stroke, as assumed previously^{5,9}. Both the rate of reversal of the working stroke and the force level achieved at its completion have therefore been underestimated in previous studies. For length steps of about 5 nm, working stroke reversal occurs at about half the forward rate. The amplitude of the X-ray signal accompanying this process (Fig. 1b) is also about half that following a shortening step from the isometric state⁸, implying a smaller tilting of the myosin heads in the reverse than in the forward working stroke.

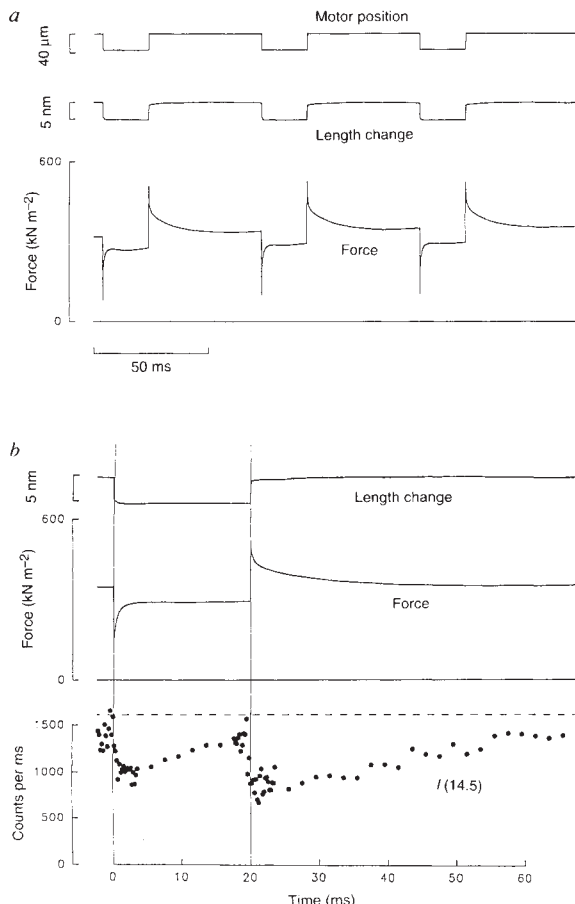


FIG. 4 Changes in force and the intensity of the 14.5 nm X-ray reflection in an active single muscle fibre subjected to a 5 nm shortening step followed 20 ms later by a 5 nm stretch, with the cycle repeated every 68 ms. *a*, Motor position, segment length change in nm per half sarcomere and force during the first three cycles of a series of 40 given during a 3 s tetanus, starting 0.25 s after the start of stimulation. Force before the 1st, 3rd, 6th and 40th shortening steps was 299, 323, 336 and 336 kN m⁻², respectively. A slightly smaller force increase was also seen in the period of an isometric tetanus corresponding to the first six cycles of this protocol. Cross-sectional area of fibre, 21,900 μm²; initial fibre length, 7.32 mm; initial mean sarcomere length in the 2.34 mm segment interrogated by the X-ray beam, 2.19 μm. *b*, Segment length change, force and the intensity of the 14.5 nm X-ray reflection (*I*(14.5)) showing the average response to the 40 cycles. The segment length and force traces are from the third cycle in *a*. The X-ray intensity data, recorded in 0.2 and 2.0 ms timeframes, are from a total of 47 tetani in 7 fibres, including the one in *a*. In three of the fibres (23 tetani), a fast shutter closed during the 2.0 ms sampling periods, and the data for these periods have been scaled accordingly. In the accumulated data from 40 cycles the intensity before the shortening and stretching steps was 87% and 85%, respectively, of the isometric level (horizontal broken line). The vertical lines denote the midpoints of the 0.12 ms length steps.

The rate of detachment and repriming deduced from the X-ray signal is less after stretch than after shortening (Fig. 4b), indicating a lower detachment rate for heads that had been strained by the 5 nm stretch, as postulated in conventional contraction models^{1,10,11}. This difference may be related to the lower ATPase during stretch than shortening^{16,17}. However, the rate of repriming after stretch in the present conditions, about 30 s⁻¹, is much larger than the ATPase rate during steady stretching which is less than 3 s⁻¹ per myosin head¹⁷. Thus, as argued previously for shortening¹², if a large fraction of the myosin heads in a fibre are involved in force generation, repriming does not commit them to ATP hydrolysis. □

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Imaging of single fluorescent molecules and individual ATP turnovers by single myosin molecules in aqueous solution

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VISUALIZATION of single actin filaments by fluorescence microscopy¹ led to the development of new *in vitro* assays for analysing actomyosin-based motility at the molecular level^{2–5}. The ability to manipulate actin filaments with a microneedle^{6,7} or an optical trap⁸ combined with position-sensitive detectors has enabled direct measurements of nanometre displacements and piconewton forces exerted by individual myosin molecules. To elucidate how myosin generates movement, it is necessary to understand how ATP hydrolysis is coupled to mechanical work at the level of the single molecule. But the most sensitive microscopic ATPase assay available still requires over 1,000 myosins⁹. To enhance the sensitivity of such assays, we have refined epifluorescence and total internal reflection microscopies to visualize single

fluorescent dye molecules. We report here that this approach can be used directly to image single fluorescently labelled myosin molecules and detect individual ATP turnover reactions. In contrast to previously reported single fluorescent molecule imaging methods, which used specimens immobilized on an air-dried surface¹⁰⁻¹², our method allows video-rate imaging of single molecules in aqueous solution, and hence can be applied to the study of many types of enzymes and biomolecules.

Single fluorescent dye molecules, excited by a relatively strong light source such as a laser, emit sufficient photons to be visualized under a conventional microscope with a commercial high-sensitivity video camera¹³. However, imaging single fluorescent dye molecules in solution requires extracting the signal from a huge background of scattering and luminescence. Single fluorescent dye molecules have been imaged at an air-surface interface by reducing the optical excitation volume to sub-wavelength proportions using illumination-mode near-field scanning optical microscopy^{10,11}. This method, however, is difficult to apply to real-time imaging of single protein molecules undergoing motion and ATPase reaction in aqueous solution, because the field image is acquired by scanning with a sharp optical probe for several minutes. Therefore, we developed two different types of fluorescence microscopy without using scanning probes that can be used in an aqueous environment.

Figure 1 shows a diagram of the epifluorescence microscope for visualizing single fluorescent molecules (low background epifluorescence microscopy, LBEFM). Background results from many sources including Raman scattering from immersion oil and the solution, incident light breaking through filters and luminescence arising from objective lens, immersion oil and dust. By appropriately choosing fluorescent dyes, excitation and emission wavelengths, an objective lens, dichroic mirrors, sharp-cut barrier filters, immersion oil and a quartz coverslip or slide, we have been able to reduce the background from >6,000 to 140 photons

per s per diffraction limit area ($0.25\ \mu\text{m} \times 0.25\ \mu\text{m}$) at an Ar laser power of 10 mW (see legend to Fig. 1 for details).

Evidence that single fluorescent dyes bound to myosin subfragment heavy meromyosin (HMM) could be seen by LBEFM was obtained in three different ways. (1) The fluorescent dye, Cy3 (ref. 14) was bound specifically to a reactive thiol group (SH-1) on both heads of HMM at an average dye to head molar ratio of 0.7:1. Therefore, the average molar ratio of HMM with two, one and no bound Cy3 molecules would be about 5:4:1. Fluorescence images of Cy3-HMM spread on the surface of a coverslip at very low density ($<0.13\ \text{HMMs}\ \mu\text{m}^{-2}$) appeared as bright spots (Fig. 2b). Figure 2a shows the histogram of intensities of individual fluorescent spots. Fluorescent spot intensities were quantized at intervals of about 500 photons per s. Fluorescence intensities of more than 1,500 photons per s probably result from two HMM molecules that were close together. When the incident light power was increased 10-fold, the fluorescent spots became much brighter but the number of spots observed was unchanged, indicating that the intensity of 500 photons per s is the minimum quantity (because of single fluorescent dye molecules). (2) We confirmed that the least intense spots corresponding to the minimum quantity were bleached in a single step, whereas the next most intense spots were bleached in two steps. Such stepwise photobleaching was most clearly observed with the second imaging method described below (Fig. 3b). (3) The spots observed by fluorescence microscopy correspond to individual HMM molecules shown by electron microscopy. Figure 2b shows fluorescence images of Cy3-HMM molecules; Fig. 2c shows the electron micrograph of the same field as that shown in Fig. 2b. Single HMM molecules, identified by their characteristic two-headed shape (inset), were found in positions (single arrow heads) corresponding to the least intense fluorescent spots (the first gaussian peak in Fig. 2a), or in positions (double arrow heads) of more intense fluorescent spots (the second gaussian peak in

FIG. 1 Schematic drawing of the optical microscope system for visualizing single fluorescent molecules. Optics, low background epifluorescence microscopy (LBEFM): an inverted microscope equipped with epifluorescence optics (TMD300, Nikon, Japan) was modified as follows. A beam of an Ar laser (514.5 nm) or a He-Ne laser (632.8 nm) was depolarized by passage through a $1/4\ \lambda$ plate and focused by lens (L1) on a rotating ground glass (RG) to eliminate inhomogeneity of illumination due to interference. The depolarized and random phase beam was passed through a pinhole (P) and was collimated with lens (L2). Then the beam was focused on an aperture diaphragm and was introduced to the fluorescence-less objective lens (OL; NCF Plan Apo $\times 100$; 1.4 NA; Nikon, Japan) through a dichroic mirror (D; specially made by Sigma Koki, Japan; it reflects 99.9% of the incident beam). The specimens were excited by paraxial rays with Koehler illumination. All glass optical parts, except for the objective lens, were replaced with ones made of fused silica, which emit little fluorescence. The fluorescence-less silicon oil (KF56, Shinetsu Silicon, Japan) was used as immersion oil. Background luminescence was rejected with a barrier filter (BF; 570DF30 for Ar laser and 670DF40 for He-Ne laser, Omega Optical, USA). M indicates reflection mirrors. To reduce dust contamination, which was a serious source of background noise, coverslips and slides were washed with 0.1 M KOH and ethanol and then dried in a class 1,000 clean room. Experiments were also done in the clean room. Dust in solution was removed by filtration before experiments. Total internal reflection fluorescence microscopy (TIRFM): the laser beam was incident on a quartz microscope slide through a 60° dispersion prism. The gap between the microscope slide and prism was filled with fluorescence-less pure glycerol. Incident angle at the quartz slide-to-solution interface was 68° to the normal (the critical angle is 66°). The beam was focused by a lens (L3) to be $100\ \mu\text{m} \times 200\ \mu\text{m}$ at the specimen plane. Imaging and analysis: a cooled CCD camera (Series 4200; Astromed, UK) was used for quantitative analysis of the fluorescence intensity with low temporal resolution. For observation of rapid movement or changes in fluorescence intensity, an ICCD camera (C2400-87, Hamamatsu Photonics, Japan) or ISIT camera (C2400-08 and C2400-80H, Hamamatsu Photonics) was used.

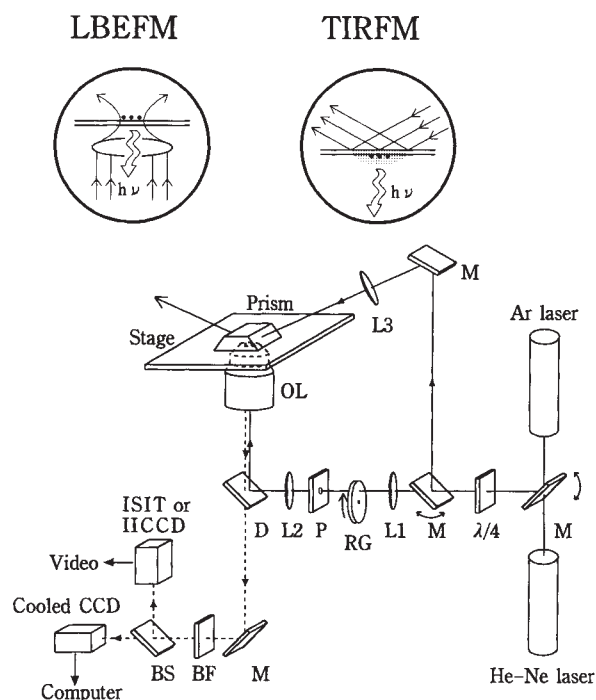


Fig. 2a). Thus, the least intense fluorescent spots are due to single fluorescent molecules attached to one of the two heads of HMM. Taken together, the evidence described above provides strong support that single fluorescent molecules can be visualized by LBEFM.

To enhance further the ability for single molecule imaging, we exploited total internal reflection optics (total internal reflection fluorescence microscopy, TIRFM)¹⁵. When a laser is incident on an interface between a quartz slide (high index) and the medium (low index) at above the critical angle, the light is totally internally reflected and the evanescent field is produced just beyond the interface in the medium with a $1/e$ penetration depth of about 150 nm. The background was $>2,000$ -fold lower than that of conventional epifluorescence microscopy (3 photons per s per diffraction limit area). Single fluorescently labelled HMM molecules could be clearly visualized at video rate (1/30 s) without frame averaging (Fig. 3a). The high-rate imaging allowed us to see clearly the time course of stepwise photobleaching of fluorescence from two dye molecules, probably bound to a single HMM (Fig. 3b).

We applied the TIRFM to detect individual Mg-ATP turnovers by single S-1 molecules (single-headed myosin subfragments) labelled with Cy5 and fixed on the quartz slide surface. The ATP turnover events were detected by directly observing association-(hydrolysis)-dissociation of a fluorescent ATP analogue labelled with Cy3. When 10 nM Cy3-ATP was applied to S-1 on the surface, the background fluorescence due to free Cy3-ATP was low, because the illumination region was localized near the quartz slide surface (Fig. 4a). When Cy3-ATP or Cy3-ADP was associated with surface-bound Cy5-S-1, which had been previously visualized (Fig. 4b), it could be seen as a clear, focused fluorescent spot (Fig. 4c); free Cy3-ATP undergoing rapid brownian motion, on the other hand, was not seen as a discrete spot. Hence, by observing the presence and lifetime of stationary, focused Cy3 molecules corresponding to the position of Cy5-S-1 molecules on the surface, we could detect individual association-(hydrolysis)-dissociation of Cy3-ATP with single S-1 (Fig. 4a). Figure 4c shows the association and dissociation of single Cy3-ATP/ADP molecules with a single S-1 molecule (indicated by an arrow head in Fig. 4b); Fig. 4d shows the

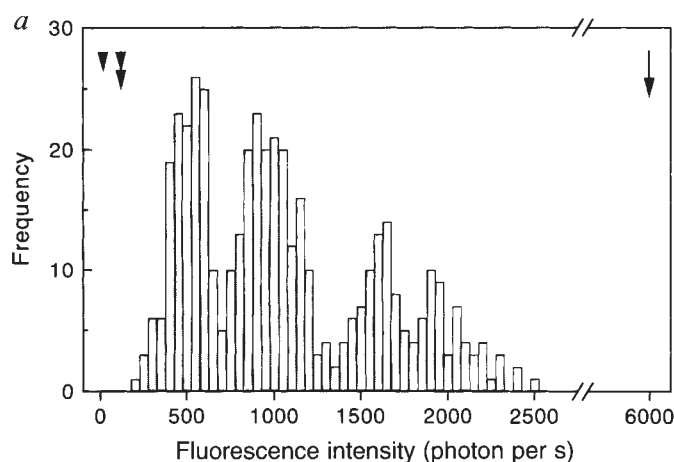
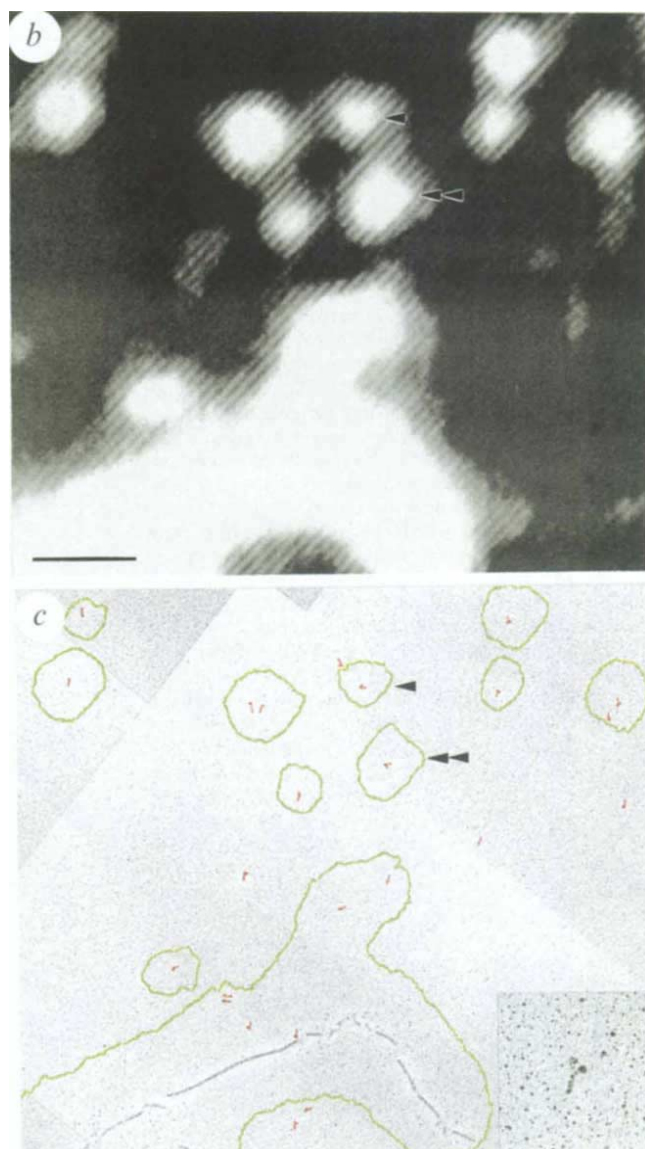


FIG. 2 Low background epifluorescence microscopy (LBEFM) imaging. a, The histogram of fluorescence intensity from Cy3-labelled HMMs. The laser power was 10 mW at the objective plane and the illumination area was 150 μ m in diameter. Images were taken with a cooled CCD at 5-s exposure. The number of photons that arrived at the camera was calculated from the number of photoelectrons and quantum efficiency ($\sim 30\%$) of the camera. The number of fluorescent spots measured was 444. The single and double arrow heads indicate the background in TIRFM and LBEFM, respectively. The arrow indicates the background before refinement. b, Fluorescence micrograph taken by LBEFM. Cy3-labelled HMMs were spread on a thin mica film to enable subsequent electron microscopy and illuminated by Ar laser. The fluorescence ($\lambda_{em} = 555\text{--}585$ nm) images were video-taped with IICCD camera and 64 frames were averaged. As a marker to identify position, phalloidin tetramethyl rhodamine-labelled actin filaments were introduced as seen in the lower left. The typical fluorescent spots due to one and two dye molecules are indicated by single and double arrow heads, respectively. Scale bar, 1 μ m. c, Rotary-shadowed electron micrograph of the same field and at the same magnification as those in b. HMM molecules are coloured red. The inset shows the high-magnification image of a HMM. The same actin filament seen by video is also seen at lower left. The outlines of the fluorescence images are shown by green lines. Some HMM molecules were located close to one another and generally corresponded to intense spots. The population of the non-fluorescent HMMs ($\sim 30\%$) was slightly higher than that estimated from the labelling ratio (10%). This is probably because some dyes were bleached before labelling reaction or during focusing an objective lens on them.

METHODS. Actin and HMM were prepared from skeletal muscle as described previously². About 70% of SH-1 of myosin heads were labelled with Cy3-maleimide obtained by linking Cy3.18-OSu (Biological Detection Systems, USA) to *N*-(2-(1-piperazinyl)ethyl)maleimide (Dojindo



Laboratories, Japan). Medium: 25 mM KCl, 3 mM $MgCl_2$, 20 mM HEPES (pH 7.8), 0.5% 2-mercaptoethanol and an oxygen scavenger system²¹.

FIG. 3 Total internal reflection fluorescence microscopy (TIRFM) imaging. *a*, The micrograph shows one frame image of Cy3-labelled HMMs taken at the video rate (exposure time, 1/30 s) without averaging. The laser power was ~ 15 mW, at which the average lifetime of fluorophores (1/photobleaching rate) was ~ 15 s. Single and double arrow heads indicate typical fluorescent spots due to one and two dye molecules bound to HMM, respectively. Lower intensity spots are also seen but are due to noise from the IICCD camera; these disappeared in frame averaged images. Scale bar, 5 μ m. *b*, Quantized photobleaching of fluorescent molecules observed at the video rate (1/30 s). a.u., Arbitrary units.

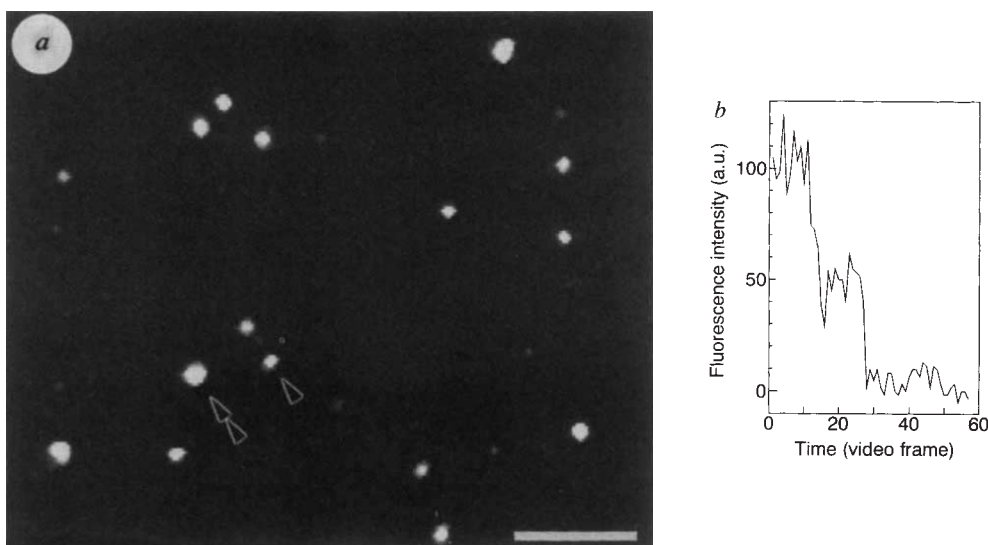
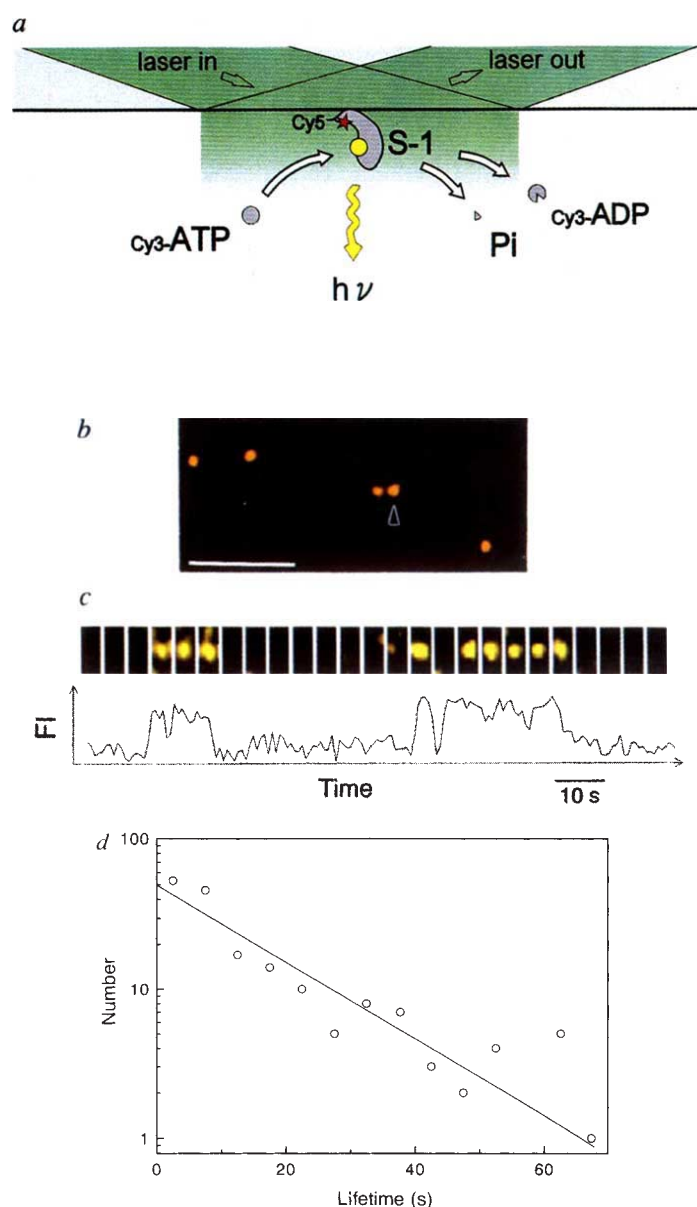


FIG. 4 Visualization of individual ATP turnovers by single S-1 molecules. *a*, The schematic of the principle of measurement (see text). *b*, Fluorescence micrograph of single Cy5-labelled S-1 molecules bound to the surface. Cy5-labelled S-1 molecules were illuminated with a 5 mW He-Ne laser and emitted red (~ 670 nm) fluorescence. The images have been artificially coloured red. Scale bar, 5 μ m. *c*, ATP turnovers by a single S-1 molecule. Upper panel shows typical images of fluorescence from Cy3-nucleotide (ATP or ADP) coming in and out of focus by associating and dissociating with an S-1 indicated by the arrowhead in *b*. Bound Cy3-nucleotides were illuminated with a 3.8 mW Ar laser and emitted yellow (~ 570 nm) fluorescence. The images have been artificially coloured yellow. Lower trace, time course of the corresponding fluorescence intensity. *d*, The lifetime of Cy3-nucleotides bound to S-1s. In the graph, the ordinate indicates the number of fluorescent spots that have some lifetime shown in the abscissa. The lifetime was determined by measuring the duration that Cy3-nucleotides bound to S-1s and made clear fluorescent spots as such as those in *c*. The number of fluorescent spots measured was 181. The dissociation rate (1/lifetime) was determined to be 0.059 s $^{-1}$ from the linear regression of the logarithmic plot.

METHODS. Preparation of Cy5-labelled S-1: Cy5-maleimide was obtained by linking Cy5.18-OSu (Biological Detection Systems) to *N*-(2-(1-piperazinyl)ethyl)maleimide. Cysteine residues of myosin regulatory light chains were labelled with Cy5-maleimide and exchanged into S-1 (ref. 22). The exchange with fluorescently labelled-regulatory light chains does not noticeably affect the function of S-1. Cy3-ATP was prepared by coupling of Cy3.29-OSu (Biological Detection Systems) with *N*⁶-((6-aminohexyl)carbamoylmethyl)-ATP²³. The reaction mixture was purified twice with an HPLC anion exchange column (monoQ, Pharmacia). Impurities could not be detected in the final elution profile of rechromatography ($<0.1\%$). Mg-ATPase activity of S-1 suspended in solution was determined by measuring P_i -release rate²⁴. The dissociation rate of bound Cy3-nucleotide (ATP or ADP) from S-1 was obtained from the ATPase rate at saturated concentration of Cy3-ATP (15 μ M), in which the ATPase rate is roughly equal to the dissociation rate. Mg-Cy3-ATPase rate of S-1 was 0.045 ± 0.002 s $^{-1}$ ($n = 2$), which was similar to that of Mg-ATPase rate (0.063 ± 0.001 s $^{-1}$, $n = 3$). Medium: 10 nM Cy3-ATP, 25 mM KCl, 3 mM MgCl₂, 20 mM HEPES (pH 7.8), 0.5% 2-mercaptoethanol and an oxygen scavenger system. All experiments were done at 25 $^{\circ}$ C.



lifetime of bound Cy3-ATP or Cy3-ADP which reveals an exponential dissociation rate, k_{-} , of 0.059 s^{-1} . The photobleaching of Cy3-ATP hardly affects the results as its lifetime (1/photobleaching rate), when bound to a quartz surface, was $\sim 85\text{ s}$ at the same laser power (3.8 mW). The dissociation rate agrees well with the ATP turnover rate of Cy3-ATP by S-1 suspended in solution ($0.045 \pm 0.002\text{ s}^{-1}$), suggesting that the binding and dissociation of Cy3-ATP/ADP visualized by microscopy indeed reflects a single ATP turnover. Because individual S-1 molecules can turn over Cy3-ATP during several minutes of observation, illumination of Cy3-nucleotide bound to S-1 does not appear to diminish enzymatic activity.

These methods can be extended to simultaneous measurements of the ATPase reaction and force/movement by single myosin molecules. This should provide a clear answer to the fundamental problem of how mechanical reaction is coupled to ATPase reaction^{16,20}. Furthermore, the methods can be applied to examining single nucleotidase reactions of other enzymes, interactions of polymerases and helicases with DNA, and a variety of protein-protein or ligand associations at a single molecular level. □

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Chemical structure of sterols that activate oocyte meiosis

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GNADOTROPHINS and various growth factors, but not sex steroids, can induce resumption of meiosis *in vitro*, but only in oocytes enclosed by cumulus-granulosa cells¹. Follicular purines prevent resumption of meiosis^{2,3}. This process can be overcome, *in vitro*, by a transient elevation of cyclic AMP resulting in the production of a diffusible meiosis-inducing substance secreted by the cumulus cells⁴. A meiosis-inducing activity has been detected in gonads of different species, for example, in preovulatory follicular fluid of women⁵ and in mouse testes⁶. We report here the isolation and characterization of meiosis-activating sterols from human follicular fluid and bull testes and the synthesis of two closely related C₂₉ sterols. All these sterols induce a resumption of meiosis in cultured cumulus-enclosed and naked mouse oocytes indicating their nonspecificity across species and sex. This family of sterols is for the first time considered crucial to meiosis.

A diffusible meiosis-regulating substance has been described in a gonad-assay consisting of cultured fetal mouse gonads⁷. A meiosis-inducing substance termed MAS (meiosis-activating sterol) to avoid confusion with Müllerian inhibitory substance⁸, also known as anti-Müllerian hormone⁹ is present when meiosis occurs in germ cells of cultured fetal mouse testes; a meiosis-preventing substance (MPS) is present when meiosis is inhibited in germ cells of cultured fetal mouse ovaries. In addition, MAS activity has been measured by the oocyte assay method as the ability to induce the resumption of meiosis in mature mouse oocytes, cumulus-enclosed oocytes and naked oocytes, overcoming the inhibition of supplemented hypoxanthine, *in vitro*⁴.

TABLE 1 Ionic species in the mass spectra of T-MAS, FF-MAS and lanosterol

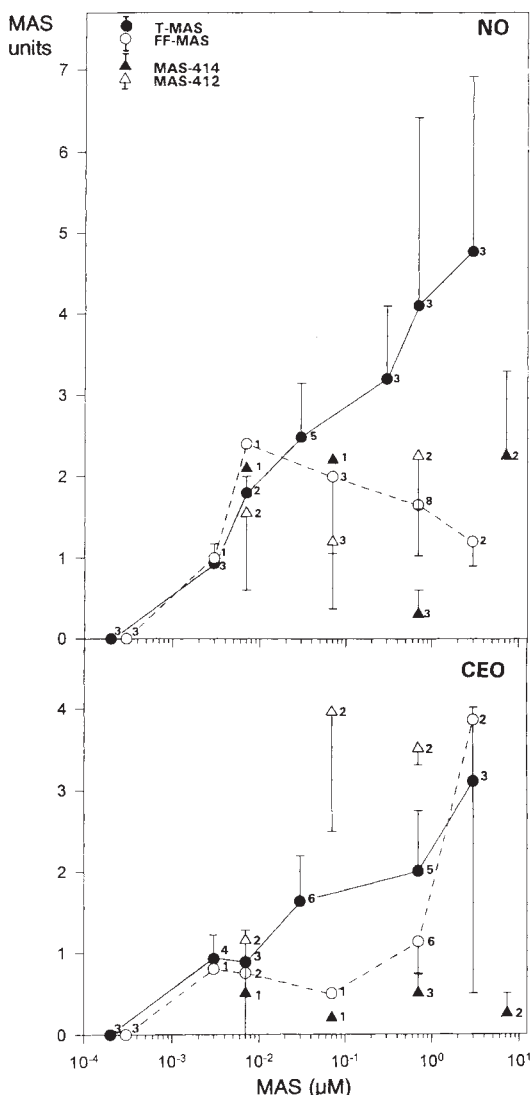
Fragmentation	T-MAS	FF-MAS	Lanosterol
[M] ⁺	412	410	426
[M-CH ₃] ⁺	397	395	411
[M-H ₂ O] ⁺	394	392	408
[M-CH ₃ -H ₂ O] ⁺	379	377	393
[M-43-H ₂ O] ⁺	351	349	365
[M-SC-2H] ⁺	299	297	313
[M-SC-H ₂ O] ⁺	283	281	297
[M-SC-42-H ₂ O] ⁺	241	239	255
[M-SC-56-H ₂ O] ⁺	227	225	241

SC, Sterol side chain (=111); 42, C₃H₆; 43, C₃H₇; 56, C₄H₈.

Characteristic prominent peaks and their M_r from the mass spectra of T-MAS, FF-MAS and lanosterol are listed. The fragmentation patterns and the molecular ions [M]⁺ are given. For purification methods of T-MAS and FF-MAS, see Fig. 2A.

The lipophilic MAS-material was extracted with *n*-heptane from human follicular fluid (FF-MAS) obtained from women undergoing treatment for infertility by *in vitro* fertilization and from bull testicular tissue (T-MAS). The MAS was purified in three consecutive high-performance liquid chromatography (HPLC) steps and each fraction was tested for activity using the oocyte assay (Fig. 1). The chromatogram from the third HPLC step had only one major peak with a retention time (t_R) = 4.04 min for T-MAS (Fig. 2A, a) and t_R = 4.18 min for FF-MAS (Fig. 2A, c). The subsequent oocyte assays of all fractions showed that only this major peak was positive (Fig. 1). An authentic standard of lanosterol was found to coelute with T-MAS (t_R = 4.03) (Fig. 2A, b), whereas T-MAS and FF-MAS did not coelute (Fig. 2A, d). Lanosterol is, however, negative in the oocyte-assay. Mass spectrometry (MS) of lanosterol, T-MAS and FF-MAS revealed that their spectra (Fig. 2B) and fragmentation patterns (Table 1) are mutually different. The relative molecular mass (M_r) of T-MAS is 412, the M_r of FF-MAS is 410, whereas lanosterol's M_r is 426. Small amounts of lanosterol may be present as an impurity in the isolated T-MAS fraction.

The structure of T-MAS was determined by nuclear magnetic resonance (NMR) primarily from its ¹³C-NMR chemical spectrum and its M_r of 412. The ¹³C-NMR chemical shifts of



T-MAS are essentially identical to either those of lanosterol¹⁰ or zymosterol¹¹ (Table 2). Because ¹³C-NMR chemical shifts are in general only weakly affected by structural modifications which occur distal to the carbon atom under observation, the structure of T-MAS was proposed to be a sterol with 29 carbon atoms (C₂₉ sterol) with the formula 4,4-dimethyl-5 α -cholest-8,24-diene-3 β -ol, also termed 4,4-dimethyl-zymosterol. T-MAS is structurally very homologous to both lanosterol (C₃₀ sterol) and zymosterol (C₂₇ sterol) in a way suggested by the tentative assignments of the various ¹³C-NMR resonances.

The MS of the compound responsible for the FF-MAS-active peak revealed that the chemical structure is 4,4-dimethyl-5 α -cholest-8,14,24-triene-3 β -ol, a C₂₉ sterol (known as trienol) (Fig. 2B). Sufficient material for NMR studies of FF-MAS has not yet been collected.

Both C₂₉ sterols, T-MAS from the bull and FF-MAS from the human, can induce a resumption of meiosis in mouse oocytes *in vitro*, and their activity therefore seems to be nonspecific across species as well as sex.

In addition, two sterols with close resemblance to T-MAS and FF-MAS have been chemically synthesized, 4,4-dimethylcholesta-8-en-3 β -ol (*M_r* 414), termed MAS-414, and 4,4-dimethylcholesta-8,14-dien-3 β -ol (*M_r* 412), termed MAS-412. These sterols also induced the resumption of oocyte meiosis (Figs 1 and 2B).

This is the first identification, to our knowledge of a family of related sterol molecules which induces a resumption of oocyte meiosis, in naked as well as in cumulus-enclosed oocytes. The

FIG. 1 MAS-activity of the third HPLC step purified fractions from bull testicular tissue (T-MAS), human follicular fluid (FF-MAS) and two closely related chemically synthesized sterols, MAS-412 and MAS-414, tested in the oocyte assay. The sterols are tested in dilutions between 7.0 μ M and 0.0003 μ M on cumulus-enclosed oocytes (CEO) and naked oocytes (NO) (number of tests shown).

METHODS. Oocyte assay: the methods used for the mouse oocyte assay have been published^{2,4}. Briefly, CEO and oocytes freed from cumulus cells (NO) were cultured for 20 h. Each test consisted of 40 oocytes, and 1 control (40 oocytes) was always run simultaneously with 3 tests. For the FF-MAS data of this figure 1,300–1,400 oocytes were used, 2,500–2,600 oocytes for the T-MAS, 480 oocytes for testing the MAS-414 and 510 oocytes for the MAS-412. The percentage of oocytes with GVBD per total number of oocytes was calculated at the end of the culture period. One unit of MAS activity was defined as:

$$1 \text{ U} = \frac{\% \text{GVBD}_{\text{control}}}{2}$$

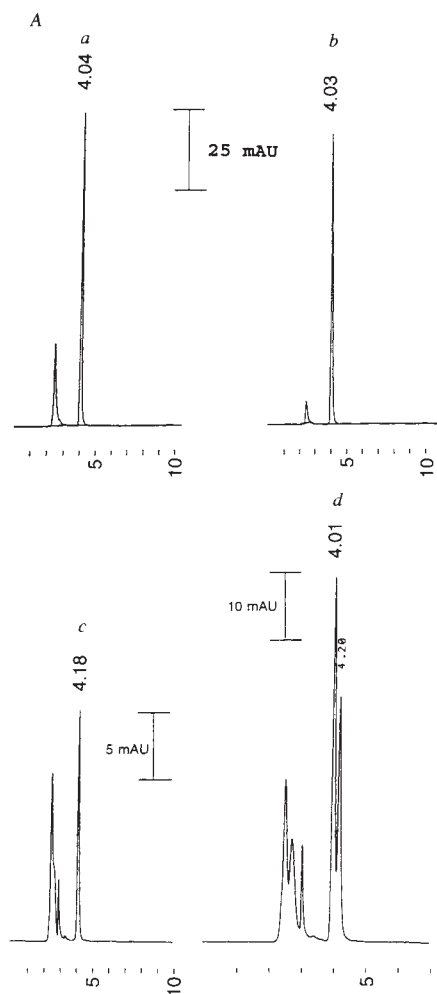
and the number of units of a test was calculated as:

$$U = 2 \times \left(\frac{\% \text{GVBD}_{\text{test}} - \% \text{GVBD}_{\text{control}}}{\% \text{GVBD}_{\text{control}}} \right)$$

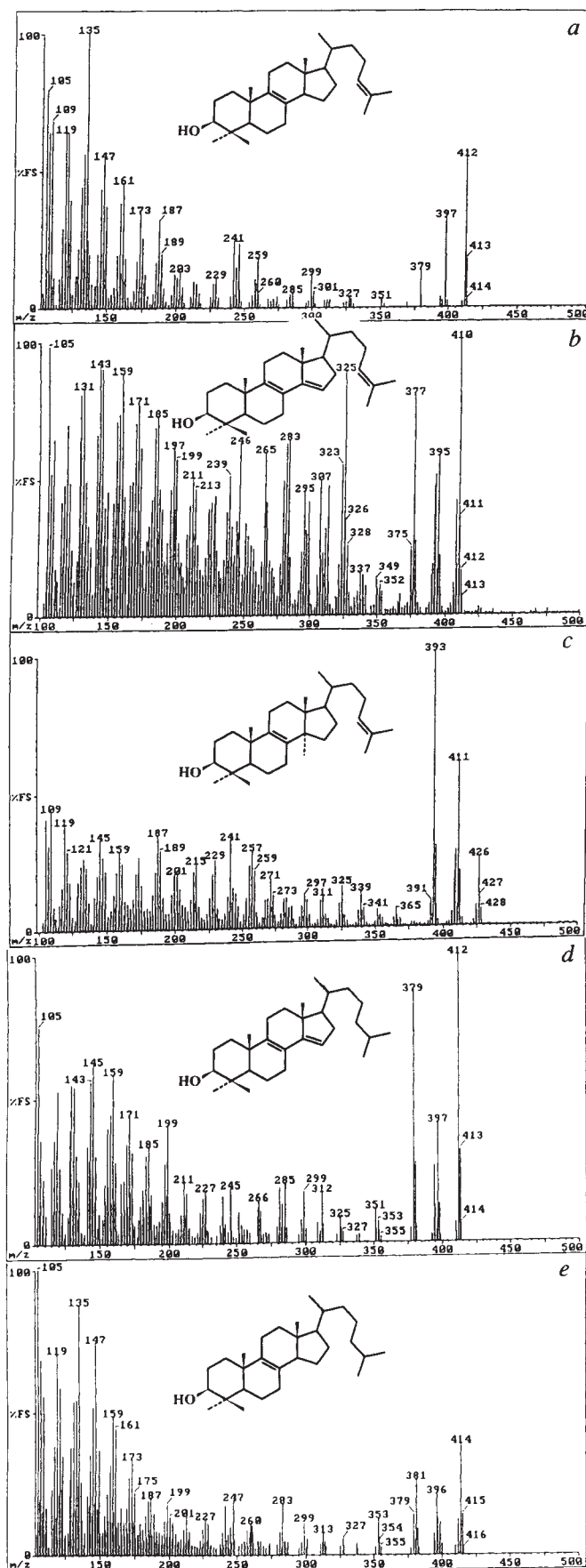
Preparation of test material: from each HPLC fraction, 0.5 ml was evaporated to dryness under nitrogen and immediately used for testing. The dried material was reconstituted and diluted with 1 ml 4 mM HX-medium⁴ with 0.5% human serum albumin (HSA). The material could not be dissolved in alcohol and was therefore dispersed in the medium using three ultrasound treatments each of 1-min duration on ice. HSA was added to facilitate the dissolving/dispersing of the lipid in the aqueous phase. The control media were treated as the test media. Fewer tests were done on FF-MAS and the two synthesized sterols than on T-MAS because of limited material. For the dose-response curve the pools of the active fractions were dried and weighed and the dilutions made from this material. Synthesis of MAS-414, and MAS-412: 4,4-dimethylcholesta-8,14-en-3 β -ol (MAS-412) was prepared as described in ref. 23. These molecules were processed further as described in ref. 16 to produce 4,4-dimethylcholesta-8-en-3 β -ol (MAS-414). Both compounds were characterized by MS, ¹H-NMR and ¹³C-NMR, and showed physical data as expected.

FIG. 2 A, Chromatograms of the third HPLC purification step of MAS and chemical structures of the sterols. a, T-MAS (4,4-dimethyl-5 α -cholest-8,24-diene-3 β -ol). The active compound eluted with *t_R* = 4.04. b, Authentic standard of lanosterol (4,4,14 α -trimethyl-5 α -cholesta-8,24-diene-3 β -ol) (*t_R* = 4.03) coelutes with the MAS-active peak. Lanosterol is, however, not MAS-active (tested on 720 oocytes, from 24 μ M to 0.007 μ M). c, FF-MAS (4,4-dimethyl-5 α -cholest-8,14,24-triene-3 β -ol). MAS-activity was found in the peak at *t_R* = 4.18. d, Mixture of the MAS-active peaks from T-MAS and FF-MAS shows that the two molecules elute differently, indicating that FF-MAS is more hydrophobic than T-MAS. B, Mass spectra of the third HPLC step MAS-active fraction from bull testicular tissue (T-MAS) (a), *M_r* 412, human follicular fluid (FF-MAS) (b), *M_r* 410, authentic standard of lanosterol (c), *M_r* 426 (Sigma), 4,4-dimethylcholesta-8,14-dien-3 β -ol (synthetic) (d), *M_r* 414 and 4,4-dimethylcholesta-8-en-3 β -ol (synthetic) (e), *M_r* 412.

METHODS. A, frozen testicular tissue (92 g, -80 °C) from a breeding bull was minced, freeze-dried in the dark (~90 h) and subjected to extraction using 400 ml *n*-heptane (4390 LiChrosolv, Merck, Germany) on a magnetic stirrer for 2 $\times$ 24 h at 20 °C. The organic phases were evaporated to dryness on a rotation evaporator at 35 °C (981 mg of extracted material). This material was dissolved in *n*-heptane and evaporated to dryness and stored under N₂ at 4 °C in the dark. Follicular fluid (FF) from women treated for infertility by *in vitro* fertilization was collected at the time of oocyte aspiration²⁴. The FF was centrifuged at 200g and stored as a pool at -20 °C. A 380 ml portion of the FF pool was extracted with 760 ml *n*-heptane in the dark at room temperature for 15 h. The organic phase was collected and treated as described above resulting in 164 mg of extracted material. A three-step HPLC purification was used on extractions from both testicular tissue and follicular fluid. The first HPLC purification: the content of each vial was dissolved in tetrahydrofuran (THF) to obtain a concentration of material of 200 mg ml⁻¹. An equal amount of distilled H₂O was added and the solution was applied to the reversed-phase HPLC column (LiChrospher



B



100 RP-8 endcapped 5 μm , 250 $\times$ 4 mm inner diameter, Merck). Mobile phase: linear gradient of THF going from 50% to 100% in 15 min (flow: 1 ml min⁻¹) at 40 °C. Eighteen fractions of 1 ml were collected and tested for MAS activity in the oocyte assay (~10,000 oocytes). The second HPLC purification: fractions with MAS activity in the oocyte assay from the first HPLC purification were dissolved in 50–100 μl 70% THF and applied to the second HPLC purification step using the same column as above. Mobile phase: linear gradient of THF going from 60% to 78% in 16 min (flow: 1 ml min⁻¹) at 40 °C. Fractions containing each peak value were collected manually and tested for MAS-activity in the oocyte assay (~6,000 oocytes). The third HPLC purification: fractions with MAS activity in the oocyte assay from the second HPLC were dissolved in 100 μl *n*-heptane:isopropanol, 98:2 (v/v) and applied to the straight phase semipreparative HPLC column (Chromspher 5 μm , 250 $\times$ 10 mm i.d. column (Chrompack). Mobile phase: *n*-heptane:isopropanol, 98:2 (v/v), flow: 5 ml min⁻¹, at room temperature. Five fractions each containing a peak were collected manually and tested for MAS activity (~7,000 oocytes, including those used for the data in Fig. 1). A UV detector (220 nm) was used in all three HPLC steps (L-4000, Merck/Hitachi). B, Mass spectra were obtained using a VG Trio 1000 LC/MS with LINC particle-beam interface (Fissons Instruments) connected to a straight-phase HPLC system consisting of a Chromspher Si 3 μm , 100 $\times$ 4.6 mm i.d. column (Chrompack). Mobile phase: *n*-heptane:2-propanol, 98:2 (v/v), flow: 0.6 ml min⁻¹, at room temperature. The MAS-active fractions were evaporated to dryness under nitrogen and redissolved in 100 μl *n*-heptane; 10–30 μl were injected into the HPLC system. The mass spectrometer was operated in the positive electron impact ionization mode with an electron energy of 70 eV. The HPLC-purity of 4,4-dimethylcholesta-8-en-3 β -ol (MAS-414) and 4,4-dimethylcholesta-8,14-dien-3 β -ol (MAS-412) were found to be 97.8% and 98.5%, respectively. UV wavelength was 220 nm and the eluent was *n*-heptane:2-propanol, 99:1. The flow rate was 1.0 ml min⁻¹. The mass spectrum signal revealed only one peak for each substance.

closely related sterols lanosterol (C_{30} sterol) and ergosterol (C_{28} sterol) and the steroid cholesterol (C_{27} steroid) were inactive in the oocyte assay in concentrations from $7\text{ }\mu\text{M}$ to $0.003\text{ }\mu\text{M}$.

The 4,4-dimethyl-zymosterol has already been characterized¹² and biosynthesized with yeast¹³ and mammalian liver cells^{14,15} and also chemically synthesized¹⁶. In yeast microsomes, lanosterol is converted to 4,4-dimethyl-zymosterol via trienol under the influence of a cytochrome P-450 reductase¹⁷. Because follicle-stimulating hormone (FSH) stimulates the NADPH-cytochrome P-450 reductase in cultured granulosa cells¹⁸ it is likely that the preovulatory peak level of FSH helps the conversion of lanosterol. FF-MAS (trienol) may thus accumulate locally within the preovulatory follicle, overcome the inhibitory influence of hypoxanthine and, in a paracrine way, cause the resumption of meiosis.

No direct physiological functions of 4,4-dimethyl-zymosterol (T-MAS), trienol (FF-MAS) and 4-methyl-zymosterol have previously been reported. However, our results strongly indicate that these sterols play a crucial role in the resumption of meiosis in mammals. The direct stimulation of the naked oocytes infer that receptors for MAS are present in the oocyte.

In mouse and pig oocytes germinal vesicle breakdown (GVBD) is inhibited with high concentrations of both oestrogens and androgens^{19,21}. The large amount of steroids produced

by the follicle mainly originates from cholesterol, delivered to the follicle cells by lipoproteins in the blood. However, the granulosa cells are also able to synthesize *de novo* sterol and steroid from acetate²², including MAS as a natural cholesterol-precursor¹⁶. Further studies are obviously needed to elucidate the synthesis and function of MAS in both sexes.

We have isolated two C_{29} sterols, one from human follicular fluid and another closely related from adult bull testis, and synthesized two closely related C_{29} sterols. All four sterols induce resumption of oocyte meiosis in the mouse, indicating that a particular family of sterols, which seem to be nonspecific across sex and species, play a role in the control of oocyte meiosis. Studies in progress will elucidate their role in meiosis of the male germ cells. This sterol family may prove to be important in the biology of reproduction and may become pertinent to new developments in contraception and treatment of infertility. □

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A role for retinoblastoma protein in potentiating transcriptional activation by the glucocorticoid receptor

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THE *Saccharomyces cerevisiae* SNF2/SWI2 protein is essential for the regulated expression of a variety of genes^{1,2}. A human SWI2/SNF2 homologue, hBm, is a positive participant in glucocorticoid-receptor-mediated transcription³, but its mechanism of action is not known. The retinoblastoma protein, RB, has also been shown to stimulate the transcription of several genes^{4–10}, although the target for RB has not been identified in any of these

TABLE 2 The ^{13}C -NMR chemical shifts of the individual carbon atoms reported for zymosterol, lanosterol and T-MAS

Carbon	Zymosterol	Lanosterol	T-MAS
1	35.1	35.5	35.8
2	31.5	27.8	28.0
3	70.9	79.0	79.0
4	38.2	38.9	38.9
5	40.7	50.4	50.2
6	25.5	18.2	18.4
7	27.1	26.5	28.5
8	128.0	134.4	128.0
9	134.8	134.4	135.8
10	35.6	37.0	37.0
11	22.8	21.0	22.1
12	36.9	30.9	29.7
13	42.0	44.4	42.1
14	51.8	49.8	51.9
15	23.7	30.8	23.8
16	28.7	28.2	28.8
17	54.7	50.4	54.8
18	11.2	15.7	11.3
19	17.8	19.1	19.8
20	36.0	36.2	36.0
21	18.6	18.6	18.6
22	36.0	36.3	36.1
23	24.7	24.9	24.8
24	125.0	125.2	125.2
25	130.6	130.9	130.9
26	17.6	17.6	17.6
27	25.7	25.7	25.7
28		15.4	15.4
29		27.9	27.9
30		24.2	

On the basis of the ^{13}C -NMR spectrum and M_r of 412 daltons, T-MAS has been identified as 4,4-dimethyl-5 α -cholest-8,24-diene-3 β -ol, also called 4,4-dimethyl-zymosterol. The observed proton chemical shifts, coupling constants and TOCSY correlations fully support the structure of T-MAS as 4,4-dimethyl-zymosterol. Two additional compounds, ~10% lanosterol, which is inactive in the oocyte assay, and 1% of a substance of unknown chemical structure, were found in the sample. About 1 mg MAS material from pools of the third HPLC purification step was dissolved in 0.6 ml chloroform- d_1 . A ^{13}C proton decoupled NMR spectrum, a ^1H NMR spectrum (with and without resolution enhancement) and a proton 2D TOCSY spectrum were recorded on a BRUKER AMX2 400 NMR spectrometer equipped with an inverse broad band 5 mm probe head with a gradient coil.

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transcriptional events. Here we show that RB upregulates glucocorticoid-receptor-mediated transcription. The effect of either RB or hBrm is dependent on the presence of the other. Furthermore, we demonstrate that RB and hBrm interact with one another *in vitro* and *in vivo*. These results highlight a new role for RB, which is to interact with hBrm in order to potentiate glucocorticoid-receptor-activated transcription.

Visual examination of the deduced polypeptide sequences of the human homologues of SWI2/SNF2, hBrm (ref. 3) and BRG1 (ref. 11), revealed that both contain the consensus RB-pocket binding motif¹² that is conserved among several viral oncoproteins (Fig. 1a). Thus RB could potentially interact with hBrm and influence its participation in glucocorticoid-receptor(GR)-mediated transcriptional events³. We first investigated whether the conserved motif could confer hBrm with the ability to bind RB. Accordingly, *in vitro* translated hBrm or the consensus site mutant hBrm*(C1292→A) was tested for retention by immobilized glutathione *S*-transferase(GST)-RB fusion proteins. The results shown in Fig. 1b demonstrate that RB and hBrm can interact directly with each other *in vitro* and that this interaction requires the RB-pocket domain and the consensus RB-binding motif of hBrm. RB and hBrm also interact at cellular concentrations when transiently expressed in COS-7 cells (Fig. 1c). This interaction was also dependent on an intact RB-pocket domain and the RB-binding motif of hBrm. The binding of RB to hBrm in a transcriptionally active microenvironment within the nucleus of intact cells was assessed with the yeast two-hybrid system¹³, where the interaction of two proteins can be related to the expression of a *lacZ* reporter gene. Results in Table 1 demonstrate that the LXCXE-sequence-mediated interaction of hBrm with the pocket domain of RB could occur within the yeast nucleus.

The *in vivo* relevance of this interaction was established through co-immunoprecipitation of endogenous RB and hBrm.

FIG. 1. hBrm bears a functional RB-binding motif.

a, Amino-acid sequence alignment of hBrm, BRG1 and the RB-binding domains of SV40 large T, adenovirus 5 E1A, HPV-16 E7 and CRPV E7. The boxed residues on the right constitute the EX₂LXCXE consensus for binding to RB^{12,22}, whereas those on the left are homologous to the E1A domain required for binding p300 (ref. 23). The hBrm and BRG1 sequences shown are from residues 1,263 to 1,301, and 1,295 to 1,333, respectively. b, hBrm requires its LXCXE motif for binding to the pocket domain of RB *in vitro*. hBrm is bound to immobilized GST-RB (373–792) (lane 3) but not the pocket-domain mutant GST-RB*(373–792, C706→F) (lane 4). This binding was abolished by a point mutation within the LXCXE motif of hBrm*(C1292→A) (lane 6). c, RB and hBrm co-immunoprecipitate from transiently expressing COS-7 cells. When expressed together in COS-7 cells, both the haemagglutinin (HA) epitope-tagged hBrm and RB were retrieved by the HA epitope-specific monoclonal antibody 12CA5 (lanes 9 and 10). This co-immunoprecipitation of HA-hBrm and RB was abolished by single point mutations within either the LXCXE motif of hBrm (lanes 11 and 12) or the RB pocket (lanes 13 and 14).

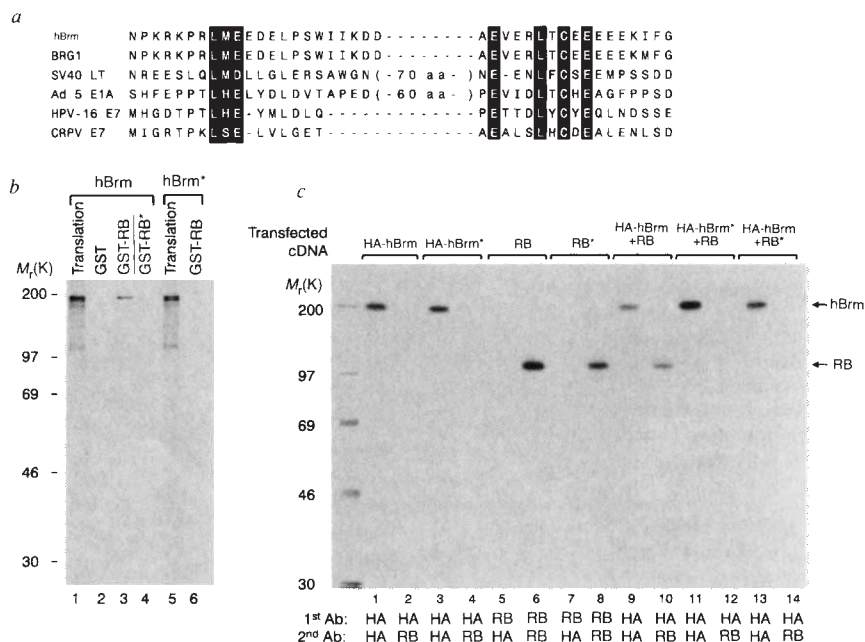
METHODS. For *in vitro* binding assays, [³⁵S]methionine-labelled, *in vitro* translated hBrm and hBrm*(C1292→A) were incubated with GST, GST-RB(373–792) or GST-RB*(373–792, C706→F) coupled to glutathione-Sepharose 4B. Binding was performed in 50 mM HEPES, pH 7.0, 150 mM KCl and 0.1% Nonidet P-40, and the matrix washed with the same buffer supplemented with 50 mM NaCl. For co-immunoprecipitation, subconfluent COS-7 cells in 80-cm² culture flasks were transfected with 12.5 µg of the cytomegalovirus-(CMV)-driven mammalian expression vector pXJ41_{neo} (ref. 24) carrying the cDNAs as indicated, and labelled for 48 h with [³⁵S]methionine.

TABLE 1 Analysis of RB binding to hBrm: relative β-galactosidase expression of double-transfected yeast cells in the two-hybrid system

DNA-binding-domain fusions	GAL4 activation-domain fusions			
	GAL4A	Gal4A-hBrm (79–1,389)	GAL4-hBrm (79–1,076)	GAL4A-hBrm (1,075–1,389)
GAL4D	1	1	1	1
GAL4D-RB	1	30	<2	40
GAL4D-RB (1–390)	1	1	1	1
GAL4D-RB (302–929)	1	36	<2	47

The region of hBrm necessary to interact with RB lies within residues 1,075–1,389, which bears the LXCXE consensus motif but excludes the bromodomain implicated in protein–protein interactions²⁵. Similarly, the region of RB necessary for interactions with hBrm encompasses the pocket domain (residues 394–773). Fusions of the GAL4 DNA-binding domain (GAL4D) with RB were created by inserting fragments of RB cDNA into pAS2 (ref. 26) and the resulting plasmids expressed in the *S. cerevisiae* strain Y190 carrying a GAL1-driven *lacZ* gene. Fusions of hBrm with the GAL4 activation domain (GAL4A) were constructed by inserting the indicated portions of the hBrm cDNA into pACT2 and the plasmids transfected into Y190 clones expressing the GAL4 fusions. Double transfectants were analysed for β-galactosidase activity as a measure of GAL1-*lacZ* expression resulting from interaction between the GAL4D-RB and GAL4A-hBrm fusions. The results are relative to clones carrying both parent plasmids, and each value represents the average of triplicate assays on four independent transfectants.

A polyclonal antibody, H290, was raised against a peptide corresponding to amino acids 290 to 307 of hBrm, a region not conserved in its homologue BRG1 (Fig. 2a). Consequently, this antibody is specific for hBrm, and does not crossreact with BRG1 (Fig. 2a, lanes 3 and 7). Extracts of the normal human



These cells were lysed in extraction buffer (50 mM Tris, pH 7.4, 150 mM KCl, 15 mM NaCl, 30 mM MgCl₂, 10 mM EGTA, 0.5% NP-40, 1 mM PMSF, 5 µg ml⁻¹ aprotinin, 78 µg ml⁻¹ benzamide and 10 µg ml⁻¹ each of antipain, chymostatin, leupeptin and pepstatin A) and immunoprecipitated with the first antibody. The retrieved proteins were eluted with 1% SDS, diluted with extraction buffer, and re-immunoprecipitated with the second antibody. RB-specific monoclonal antibody C36 and HA epitope-specific monoclonal antibody 12CA5 were used.

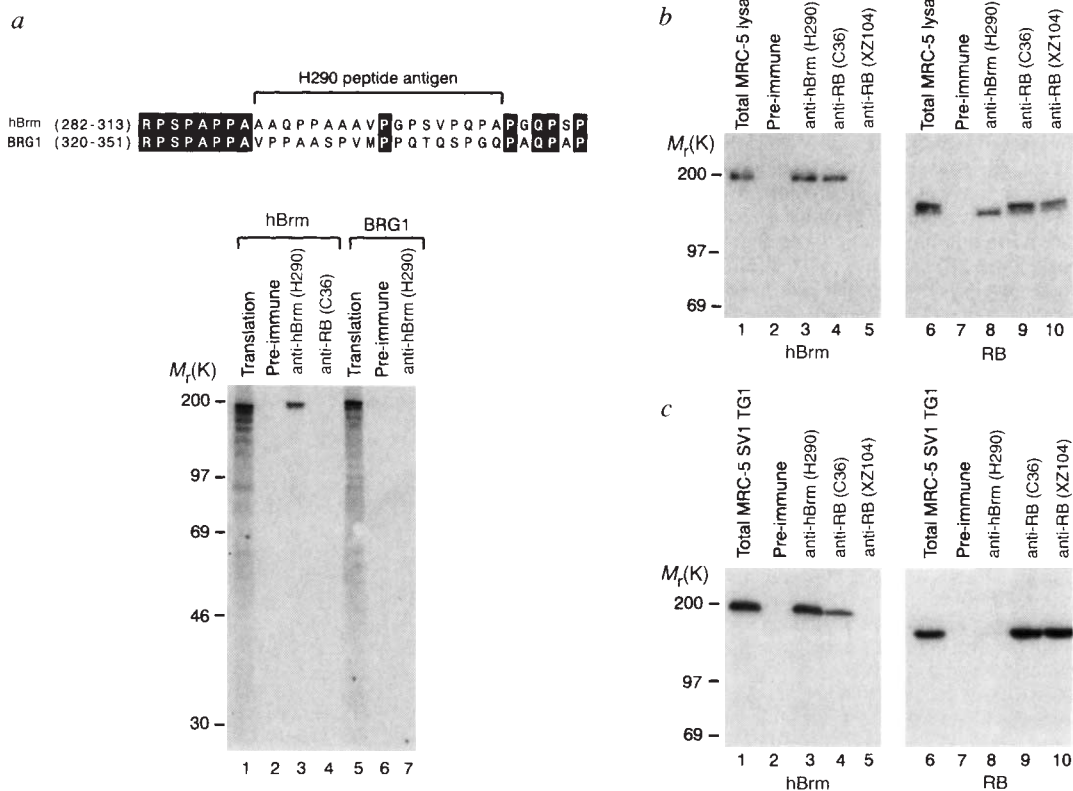
fetal lung fibroblast, MRC-5, were exposed to H290 or anti-RB antibodies, and the proteins bound by each analysed for RB or hBrm (Fig. 2b). H290 (lane 3) and the anti-RB antibody C36 (lane 4) both retrieved hBrm. Because C36 does not crossreact with hBrm (Fig. 2a, lane 4), this co-retrieval of hBrm must occur through its attachment to the bound RB. Another RB monoclonal antibody, ZX104, the recognition specificity of which includes the RB pocket, did not co-immunoprecipitate hBrm (lane 5), although both C36 and ZX104 retrieved similar amounts of RB (lanes 9 and 10). Also, RB was co-immunoprecipitated by H290 (lane 8). Although immunoprecipitation and immunoblot analysis of cell extracts with RB antibody revealed two bands (lanes 6, 9 and 10) corresponding to the differentially phosphorylated forms of RB¹⁴⁻¹⁷, only the faster-migrating hypophosphorylated form co-immunoprecipitated with hBrm (lane 8). This specificity of hBrm for the hypophosphorylated form of RB is noteworthy, as this form of RB is credited with its cellular function¹⁸ and is also the form of RB targeted by viral oncoproteins¹⁹. Similar results were obtained with other cell types (data not shown). This specific interaction between RB and hBrm was selectively reduced in MRC-5 SV1 TG1 cells that express the SV40 large T-cell antigen, a viral RB-binding protein (Fig. 2c). Hence, hBrm exhibits stable binding to hypophosphorylated RB *in vivo*, and is thus a bona fide cellular RB-binding protein.

Because hBrm is known to potentiate GR-mediated transcriptional activation, we investigated the effect of RB on this activity. HeLa cells were transfected with RB and GR complementary DNA constructs, and a chloramphenicol acetyl transferase (CAT) reporter gene with tandem glucocorticoid response ele-

ments (GRE₂). Wild-type RB potentiated the dexamethasone-induced expression of GRE₂-CAT fivefold (Fig. 3a, lane 3), whereas E2F-1-mediated expression of an adenovirus E2 promoter-driven CAT reporter was repressed (lane 7). This effect of RB, which is dose dependent and saturable (Fig. 3b), requires a functional RB-pocket domain as the mutant RB had no effect (Fig. 3a, lane 4 and b). Similar results were obtained with MRC-5 cells (data not shown). To find out whether hBrm was the mediator through which RB exerted its effects, we re-examined the phenomenon using SW13 cells, which are devoid of hBrm³. In these cells, RB alone did not affect dexamethasone-induced expression of GRE₂-CAT (Fig. 3c, lane 3). However, in the presence of transfected hBrm cDNA, RB boosted the 6-fold increase elicited by hBrm alone (lane 5) to 12-fold over basal GRE₂-CAT expression (lane 7). The mutant hBrm*(C1292→A), which fails to bind RB (Fig. 1b, lane 6), could not support the positive effect of RB (lane 9). Similarly, mutant RB did not potentiate GR transactivation (lanes 4 and 8). These results establish that RB requires hBrm to potentiate GR-mediated transcriptional activation. To determine whether the upregulation of GR activity by hBrm involves RB, GR transactivation was analysed in RB-deficient SAOS-2 cells²⁰. In these cells, hBrm alone had essentially no effect on the expression of GRE₂-CAT (Fig. 3d, lane 5). Introduction of exogenous RB increased CAT activity fivefold (lane 3), probably through interaction with endogenous hBrm. Further introduction of hBrm boosted this to eightfold (lane 7). This indicates that the positive effect of hBrm on GR-mediated transcriptional activation requires RB. To verify the requirement for both hBrm and RB, GRE₂-CAT expression was examined in the cell line C33A, which carries an

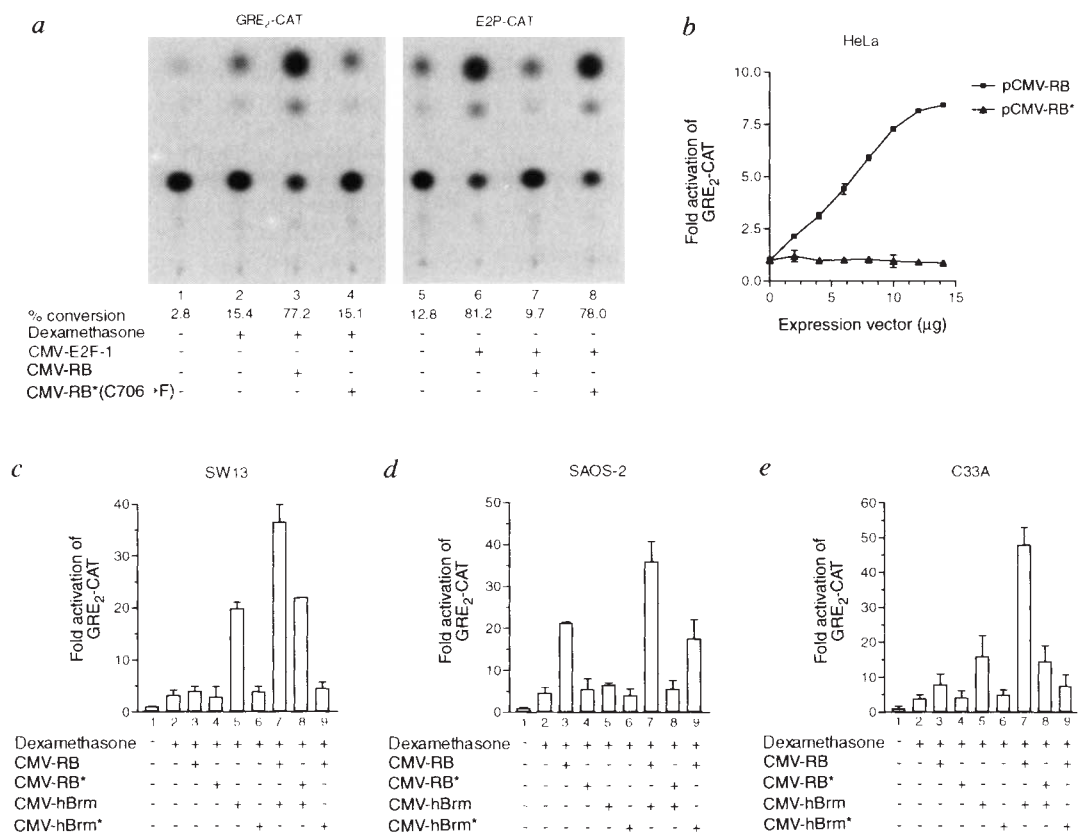
FIG. 2 hBrm binds to hypophosphorylated RB *in vivo*. a, Polyclonal antibody H290 is specific for hBrm. H290 antibody was raised against a peptide corresponding to residues 290–307 of the hBrm sequence which differs from the corresponding sequence of BRG1 as shown at the top. Its specificity for hBrm but not BRG1 was confirmed by immunoprecipitation of [³⁵S]methionine-labelled, *in vitro* translated hBrm and BRG1 in extraction buffer with the indicated antibodies. H290 specifically immunoprecipitated hBrm (lane 3) but not BRG1 (lane 7). The anti-RB antibody C36 did not crossreact with hBrm (lane 4). b and c, hBrm co-immunoprecipitates with RB. Both hBrm (b, lanes 3 and 4) and RB (lanes 8 and 9) were immunoprecipitated from MRC-5 lysates by antibodies specific for either protein. This co-immunoprecipitation was reduced by the presence of the SV40 large T antigen (c, lanes 4 and 9).

METHODS. Subconfluent MRC-5 or MRC-5 SV1 TG1 cells in 10-cm culture dishes were lysed in extraction buffer, clarified by centrifugation, and the supernatants immunoprecipitated with the polyclonal hBrm antibody H290 or the monoclonal RB antibodies C36 or ZX104. The



bound proteins were analysed by immunoblotting with H290 (left panels) or the monoclonal RB antibody G3-245 (right panels). Lanes 1 and 6 contained one-tenth of the total cell extract used for immunoprecipitation.

FIG. 3 RB cooperates with hBrm to potentiate GR-mediated transcriptional activation. *a*, RB potentiates transcriptional activation by GR. Expression of exogenous RB in HeLa cells enhanced GRE₂-CAT expression fivefold (lane 3) but suppressed expression of E2P-CAT (lane 7). *b*, Effect of RB on GR-mediated transcriptional activation is dose dependent, saturable and requires its bipartite pocket domain. HeLa cells were transfected with increasing amounts of cDNA coding for RB or the pocket domain mutant RB*(C706→F). The expression of GRE₂-CAT increased linearly with increasing RB cDNA but was unaffected by the pocket domain mutant RB*(C706→F). *c-e*, Both RB and hBrm are required for GR-mediated transcriptional activation. Significant expression of GRE₂-CAT in hBrm-deficient SW13 cells, RB-deficient SAOS-2, or hBrm, and RB-deficient C33A cells was detected only when both components were present. **METHODS.** Subconfluent cells in 60-mm dishes were transfected with the GR-expression plasmid, GRE₂-CAT reporter gene, control luciferase reporter and the indicated combinations of CMV-RB, CMV-RB*(C706→F), CMV-hBrm and CMV-hBrm*(C1292→A) expression plasmids. For lanes 5–8 of *a*, the GR-expression plasmid and GRE₂-CAT were replaced with an E2F-1 expression plasmid and a CAT reporter gene driven by the E2F-responsive adenovirus E2 promoter (E2P).



Where indicated, the transfected cells were treated with 1 μM dexamethasone for 40 h before extracts were assayed for CAT activity. Experiments in triplicate with equal amounts of total protein were normalized for transfection and extraction efficiencies by comparing luciferase activities. The specific activity of luciferase was unaffected by the transfected plasmids, and no GRE₂-CAT expression was detected in the absence of dexamethasone.

inactive pocket-domain mutant of RB²¹ and lacks hBrm³. In this cell, RB alone had no significant effect on GR-mediated GRE₂-CAT expression (Fig. 3e, lane 3), whereas hBrm gave only a fourfold potentiation (lane 5), possibly through interactions with cellular homologues of RB. However, when present together, RB and hBrm achieved a 12-fold increase in the expression of the GRE₂-CAT reporter (lane 7). Taken together, these results establish that RB can positively influence GR-mediated transcriptional activation, and confirm a similar capability for hBrm. Because the effects of hBrm and RB are dependent on

each other, and are disrupted by the same point mutations in the RB pocket domain or the RB-binding motif of hBrm that abrogate their *in vitro* and *in vivo* interactions, it can be concluded that RB exerts its effects through direct binding to hBrm.

Although RB is known to participate in the positive regulation of several genes⁴⁻¹⁰, the direct target for RB has not been defined in any of these transcriptional events. The present study establishes a new role for RB in positively regulating GR-mediated transcriptional activation through direct interaction with hBrm. □

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RPA involvement in the damage-recognition and incision steps of nucleotide excision repair

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HUMAN replication protein (RPA) functions in DNA replication¹⁻⁴, homologous recombination⁵ and nucleotide excision repair⁶. This multisubunit single-stranded DNA-binding protein^{1,2} may be required to make unique protein-protein contacts because heterologous single-stranded binding proteins cannot substitute for RPA in these diverse DNA transactions⁵⁻⁷. We report here that, by using affinity chromatography and immunoprecipitation, we found that human RPA bound specifically and directly to two excision repair proteins, the xeroderma pigmentosum damage-recognition protein XPA (refs 8, 9) and the endonuclease XPG (refs 10-13). Although it had been suggested that RPA might function before the DNA synthesis repair stage^{14,15}, our finding that a complex of RPA and XPA showed a striking cooperativity in binding to DNA lesions indicates that RPA may function at the very earliest stage of excision repair. In addition, by binding XPG, RPA may target this endonuclease to damaged DNA.

To identify proteins interacting with human RPA, all three subunits of RPA were coexpressed in and purified from *Escherichia coli* cells¹⁶ and used as ligand in affinity chromatography experiments with HeLa cell extracts. Preliminary experiments indicated that a protein with a behaviour on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and phosphocellulose (P11) chromatography similar to that of the xeroderma pigmentosum (XP) protein XPA (ref. 9) was selectively retained by RPA columns (data not shown). Knowing that RPA, like XPA, functions in nucleotide excision repair, we therefore used anti-XPA antibodies to examine whether XPA present in a P11-fractionated HeLa cell extract could bind to immobilized RPA (Fig. 1a). As the concentration of immobilized RPA was increased, there was a corresponding increase in the recovery of XPA in the fraction eluted by high salt from the RPA columns (lanes 3, 5 and 7) and a concomitant decrease in the amount of XPA present in the flow-through fractions (lanes 2, 4 and 6). To confirm the interaction between XPA and RPA, we also prepared affinity columns with immobilized recombinant XPA (ref. 17). Western blotting with a polyclonal anti-RPA serum (Fig. 1b) showed that RPA present in a HeLa cell extract was retained by the XPA column. This interaction between XPA and RPA probably occurs *in vivo*; anti-XPA antibodies could coprecipitate RPA and anti-RPA antibodies coprecipitated XPA from a HeLa cell extract (Fig. 1c).

To establish whether this interaction between RPA and XPA was direct or was mediated by other proteins in the cell extract, we chromatographed recombinant RPA on both a control and a recombinant XPA column (Fig. 1d). The XPA column (right panel), but not the control column (left panel), efficiently retained all three subunits of human RPA. This direct interaction between XPA and human RPA appeared to be species specific because yeast RPA failed to bind to human XPA (data not shown). To identify which subunits of RPA contact XPA, each

subunit of human RPA was labelled with [³⁵S]methionine by *in vitro* translation and tested for interaction with immobilized XPA. As shown in Fig. 2, an XPA affinity matrix could retain both the RPA1 (70K) and the RPA2 (32K) subunits (Fig. 2b, d). These interactions are unlikely to be mediated by DNA because *in vitro* translated yeast RPA subunits that bind DNA failed to bind to XPA columns (data not shown). Neither of these two human RPA subunits bound to control columns (Fig. 2a, c) and the 14K RPA3 polypeptide did not bind XPA (data not shown).

The role of RPA in nucleotide excision repair has generally been attributed to its abilities to bind the single-stranded DNA created by the excision of nucleotides flanking a DNA lesion, and to stimulate DNA polymerases, functions analogous to the roles of RPA in the initiation of DNA replication^{3,4}. Because the requirement for RPA in excision repair reactions *in vitro* can be bypassed by first incising damaged DNA with the *E. coli* UvrABC complex, it was suggested that RPA might also act at an earlier stage of repair rather than exclusively in repair synthesis¹⁴. The direct and specific interaction between XPA and RPA seen in our affinity chromatography experiments suggested that this interaction might affect damage recognition by XPA. By itself, XPA binds ultraviolet-damaged DNA much less well than its prokaryotic counterpart UvrA (ref. 18). Using DNA treated with the UV mimetic *N*-acetoxy-2-acetylaminofluorene (AAAF), we assessed binding of XPA and RPA to untreated and AAAF-damaged DNA (Fig. 3). Just as reported earlier with ultraviolet- or cisplatin-treated DNA (refs 9, 17), XPA bound to AAAF-treated DNA preferentially, with more XPA being sequestered to damaged DNA than undamaged DNA (Fig. 3a, b, lanes 1-4). As with cisplatin-damaged DNA (ref. 19), some preferential binding of RPA to the AAAF-treated DNA could also be detected (Fig. 3b, lanes 5-8). When both XPA and RPA were incubated together with damaged DNA, however, there was a striking cooperativity in binding DNA, the presence of both these interacting proteins increasing about 10-fold the binding of them both to AAAF-treated DNA (Fig. 3b, lanes 9-12). This enhanced recognition of damaged DNA is dependent upon the RPA-XPA interaction; *E. coli* single-stranded DNA binding protein (SSB) did not bind to XPA (data not shown), nor did it affect damage recognition by XPA (Fig. 3b, lanes 13-16). Cisplatin-treated DNA was also bound cooperatively by XPA and RPA (data not shown).

These data indicate that RPA may function with XPA at the very earliest damage recognition step in excision repair. As the next step in repair appears to be the incision made by the endonuclease XPG at the 3' side of a lesion^{12,13}, we asked if XPG might also interact with XPA or RPA. Using a co-immunoprecipitation protocol (Fig. 4a), we showed that XPG binds to RPA. [³⁵S]methionine-labelled XPG was precipitated specifically with RPA by anti-RPA (lane 6) and not preimmune serum (lane 5). In contrast, anti-XPA precipitated XPG only if RPA was present as well as XPA (lanes 4, 7), indicating that RPA mediated formation of an XPA-RPA-XPG complex. Interestingly, XPG contains an acidic region which can activate transcription when fused to the DNA-binding domain of GAL4 (ref. 20) and several different acidic transactivators make highly selective and direct interactions with RPA (refs 21-23). Indeed, this acidic region of XPG appears to mediate the XPG-RPA interaction, for when amino acids 668-747 of XPG were expressed in *E. coli* as a fusion with maltose binding protein (MBP), this MBP-XPG chimaeric polypeptide, but not MBP itself, bound human RPA (Fig. 4b, lanes 6, 8). XPA could also bind to the MBP-XPG fusion protein, but only if RPA was also present in the incubation (compare lane 4 and lane 8). A direct contact between XPG and the RPA-XPA complex bound to DNA lesions may be important in targeting the endonuclease activity of XPG to its substrate, a splayed-arm DNA structure at the duplex-single-strand junction 3' to the DNA lesion¹². An interaction between XPA and the ERCC1-XPF complex^{24,25} may similarly position this second endonuclease on DNA 5' to the lesion.

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FIG. 1 XPA binds specifically and directly to human RPA. **a**, An immunoblot of affinity column fractions with anti-XPA antibodies. Aliquots (30 μ l) of the P11 1.0 M NaCl fraction (I) loaded (lane 1) and the flow-through (FT) fraction (lanes 2, 4, 6) and one-half of the 1.0 M NaCl eluates (E) (lanes 3, 5, 7) from control (lanes 2, 3) and RPA columns (lanes 4–7) were resolved by SDS-PAGE, transferred to nitrocellulose and probed with affinity-purified rabbit anti-XPA antibodies. The concentration of immobilized RPA on each of the columns is indicated. **b**, An immunoblot of affinity column fractions with anti-RPA antibodies. Aliquots (25 μ l) of the unfractionated HeLa cell extract (I) loaded (lane 1) and the flow-through (FT) fraction (lanes 2, 4) and one-half of the 1.0 M NaCl eluates (E) (lanes 3, 5) from control (lanes 2, 3) and XPA columns (lanes 4, 5) were resolved by SDS-PAGE and probed with anti-RPA serum after transfer to nitrocellulose. **c**, Association of human RPA and XPA in a HeLa cell extract detected by co-immunoprecipitation. HeLa cell extracts were incubated with 5 μ l preimmune serum (lane 1), 5 μ l anti-XPA serum (lane 2), 0.5 μ g of a control anti-T antigen monoclonal antibody (pAb419) (lane 3), or 0.5 μ g of anti-RPA2 monoclonal antibody (71-9A, ref. 29) (lane 4). The bound proteins in the immunoprecipitates were then analysed by SDS-PAGE, and immunoblotted with either a mix of anti-RPA1 and anti-RPA2 monoclonal antibodies (lanes 1, 2) or affinity-purified rabbit anti-XPA (lanes 3, 4). The heavy bands in lanes 1 and 2 represent crossreaction of rabbit IgG heavy chain with goat anti-mouse antibodies. **d**, SDS-PAGE was used to resolve human RPA subunits present in flow-through (FT), wash (1–3), and 1.0 M NaCl eluted fractions (E) of a 20 μ l control (left panel) or XPA (2 mg ml⁻¹, right panel) column that had been loaded with 3.0 μ g purified recombinant human RPA. The gel was stained with Coomassie blue.

METHODS. Recombinant human RPA (ref. 16) and XPA (ref. 17) were expressed in and purified from *E. coli* and then coupled to Affigel 10 (Bio-Rad) at the indicated concentrations. HeLa (400 μ l) cell extract²¹ or P11-bound fraction¹⁵ was loaded at 0.1 M NaCl on 20- μ l affinity columns. After washing with 200 μ l affinity-column buffer (ACB: 20 mM HEPES, pH 7.9, 1 mM EDTA, 1 mM DTT, 10% glycerol) containing 0.1 M NaCl, the columns were eluted with 80 μ l of ACB containing 1.0 M NaCl. Polyclonal anti-XPA and anti-RPA sera were raised in rabbits using purified recombinant proteins. Anti-XPA antibodies were further affinity purified by absorption to XPA-Affigel 10. The immunoblots were developed using alkaline phosphatase-coupled goat anti-rabbit IgG. The chromatography illustrated in **d** followed a

procedure described previously²⁷. For the co-immunoprecipitation experiment shown in Fig. 1c, 1 ml aliquots of HeLa cell extract containing 0.05% Nonidet-P40 were incubated with the indicated antibodies at 4 °C, then with 25 μ l protein A/G-agarose (Santa Cruz Biotechnology) for another 2 h at 4 °C. The beads were washed 5 times with 1 ml ACB buffer containing 0.05% N-P40, and the bound proteins were eluted by ACB buffer containing 1.0 M NaCl and analysed by SDS-PAGE and immunoblotting using an enhanced chemiluminescence procedure (Amersham). With these protocols, about 1% and 4% of the total cellular RPA and XPA, respectively, was recovered.

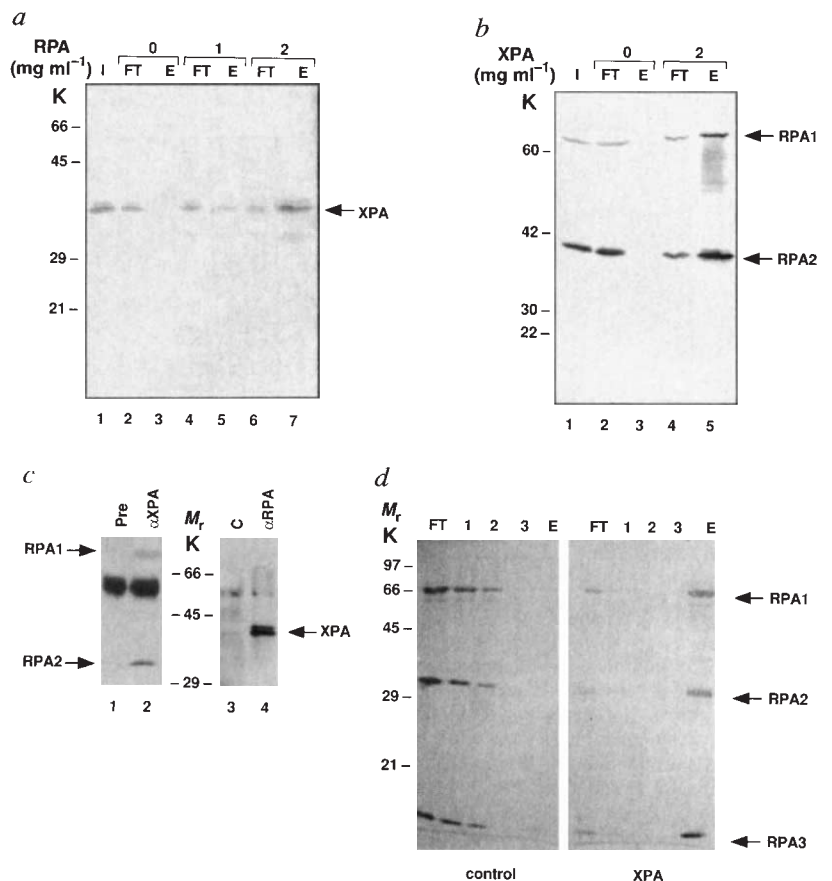
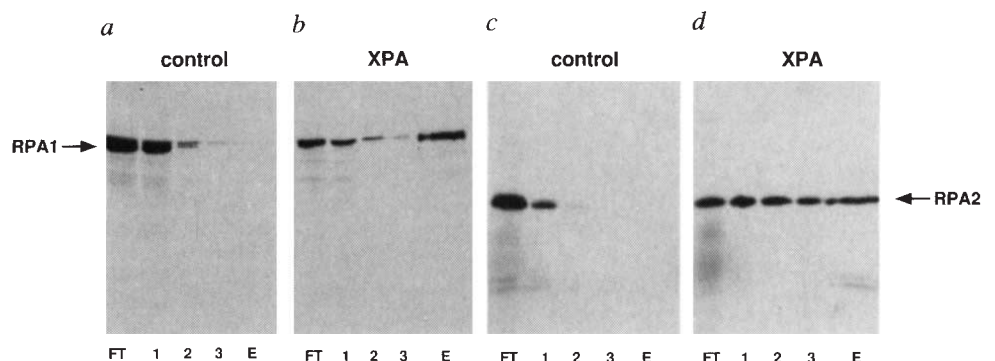


FIG. 2 Both RPA1 and RPA2 subunits of human RPA bind XPA. [³⁵S]methionine-labelled RPA1 and RPA2 were prepared by *in vitro* transcription/translation in a coupled system (Promega). Aliquots (5 μ l) of reticulocyte lysate containing either protein were mixed with 15 μ l ACB containing 0.1 M NaCl and chromatographed on 20- μ l affinity columns containing either no bound protein (**a**, **c**) or 2.0 mg ml⁻¹ XPA (**b**, **d**). The flow-through (FT), each of three 50- μ l wash fractions (1–3), and the 1.0 M NaCl eluates (E) were analysed by SDS-PAGE and fluorography. RPA1 and RPA2 were expressed from plasmids p7Oexpr (ref. 28) and pLE1 (ref. 29), respectively.



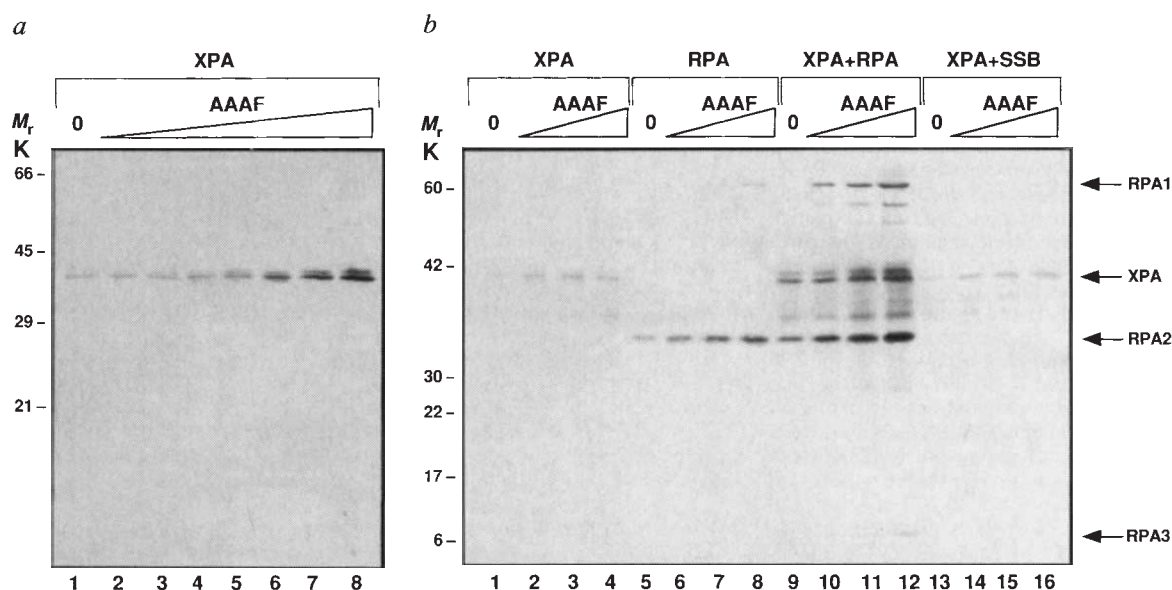


FIG. 3 RPA and XPA bind AAF-damaged DNA cooperatively. *a*, XPA preferentially binds to AAF-damaged DNA. Biotinylated DNA fragments were untreated (lane 1), or treated with 1, 2, 5, 10, 20, 30 and 50 mM (lanes 2–8) AAF, immobilized on streptavidin-beads and incubated at 4 °C for 2 h with 1.0 µg of XPA. The DNA-bound XPA was analysed by SDS-PAGE, transferred to nitrocellulose and probed with anti-XPA antibodies. *b*, Cooperative binding of damaged DNA. Biotinylated DNA fragments were untreated (lanes 1, 5, 9, 13), or treated with 5 mM (lanes 2, 6, 10, 14), 10 mM (lanes 3, 7, 11, 15), or 50 mM (lanes 4, 8, 12, 16) AAF, bound to streptavidin-beads and incubated at 4 °C for 2 h with 0.5 µg of XPA (lanes 1–4), 1.0 µg of RPA (lanes 5–8), 0.5 µg of XPA and 1.0 µg of RPA (lanes 9–12), or 0.5 µg of XPA and 0.5 µg of *E. coli* SSB (USB, lanes 13–16). The DNA-bound XPA and RPA were similarly analysed with a mixture of anti-RPA and anti-XPA sera.

METHODS. *Hind*III–*Pvu*II fragments (258 bp and 190 bp) from the plasmid pBluescript IKS (Stratagene) were 3' end-labelled with biotin-14-dATP (Gibco) using the Klenow fragment of DNA polymerase I, purified on NAP-5 columns (Pharmacia), treated with AAF for 3 h at 37 °C (ref. 30), and ethanol-precipitated twice. The resulting DNA (400 ng) was incubated with 5 µg prewashed Dynabeads M-280 streptavidin (Dyna) in 50 µl binding buffer (25 mM HEPES, pH 7.5, 50 mM KCl, 5 mM MgCl₂, 1 mM EDTA, 10% glycerol, 1 mM DTT, 0.02% Nonidet-P40) for 1 h at 22 °C. The Dynabeads were then collected by a magnetic particle concentrator (Dyna) and washed three times with binding buffer. XPA and/or RPA or *E. coli* SSB were incubated with 5 µg of DNA-bound Dynabeads in 50 µl binding buffer containing 1% BSA at 4 °C for 2 h. The bound proteins were magnetically purified, SDS sample loading buffer was added and the bound polypeptides resolved by 12.5% SDS-PAGE followed by immunoblotting.

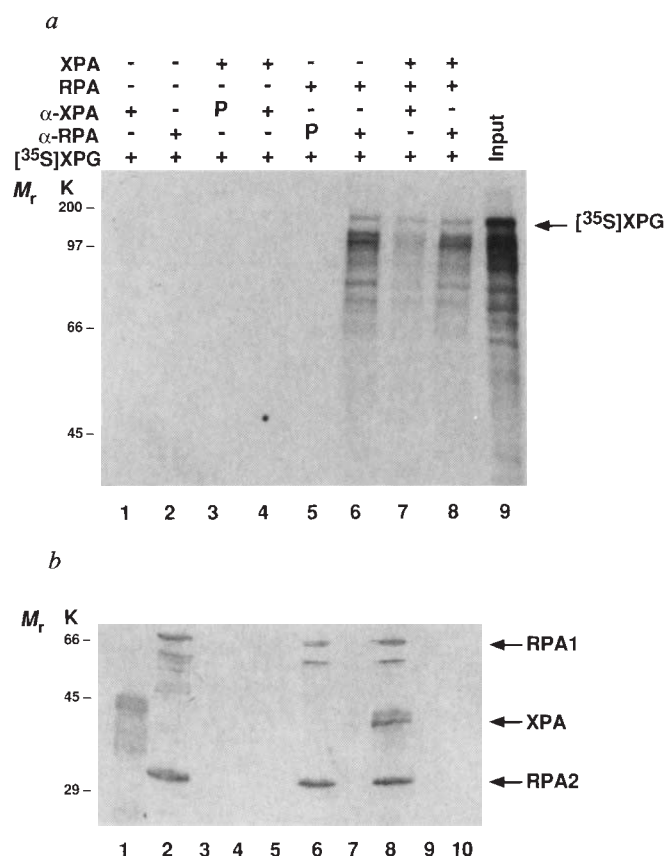


FIG. 4 RPA interacts with XPG and mediates the formation of an XPA-RPA-XPG complex. *a*, SDS-PAGE and fluorography was used to analyse the binding of [³⁵S]methionine-labelled XPG to protein A beads that had been preincubated with 5 µl of preimmune (p), anti-RPA, or anti-XPA serum and 2 µg RPA and/or 2 µg XPA as indicated. *b*, SDS-PAGE and immunoblotting was used to detect XPA and RPA proteins bound to either control MBP-amylose beads (lanes 3, 5, 7 and 9) or MBP-XPG fusion protein bound beads (lanes 4, 6, 8 and 10). The beads had been incubated with 1.0 µg of XPA (lanes 3, 4), 1.0 µg of RPA (lanes 5, 6), 1.0 µg of XPA and 1.0 µg of RPA (lanes 7, 8), or no protein (lanes 9, 10). Lanes 1 and 2, 0.25 µg of input XPA and RPA, respectively. METHODS. To examine the binding of *in vitro* translated XPG, 40 µl protein A-Sepharose beads (Sigma) were first preincubated with anti-sera, RPA and/or XPA in IP buffer (25 mM HEPES, pH 7.6, 100 mM KCl, 4 mM MgCl₂, 1 mM DTT, 12.5% glycerol, and 0.02% N-P40), washed three times, resuspended in 40 µl IP buffer and incubated with 10 µl of [³⁵S]methionine-labelled XPG for 2 h at 4 °C. After three washes with IP buffer, bound proteins were released by boiling the beads in 50 µl SDS-containing sample buffer and analysed by SDS-PAGE and fluorography. [³⁵S]XPG was synthesized by *in vitro* transcription/translation from the plasmid pRAD2synthetic kindly provided by S. Clarkson. The input [³⁵S]XPG (5 µl) is shown in *a*, lane 9. To express the acidic region of XPG as a fusion with MBP, the 236 bp *Eco*RI fragment encoding amino acids 668 to 747 of XPG from pRAD2synthetic was subcloned into pMAL-2c (NEB). The expression and purification of MBP and the MBP-XPG fusion protein followed the manufacturer's recommended protocol (NEB). The amylose beads carrying 5 µg of MBP or the MBP-XPG fusion protein were incubated with the indicated proteins in IP buffer for 2 h at 4 °C, and then washed four times with IP buffer. The bound proteins were eluted with IP buffer containing 1.0 M KCl, and analysed by SDS-PAGE and immunoblotted with a mixture of anti-XPA and anti-RPA antibodies.

Although our data suggest that RPA is involved together with XPA and XPG in the very earliest steps of excision repair, it does not exclude an involvement of RPA in the strand-displacement and DNA synthesis steps. Despite such a prominent role for RPA in excision repair, mutations in human RPA genes that affect DNA repair have not yet been identified (but see ref. 26). Mutations in RPA may be lethal because they impinge on essential steps of DNA replication. Therefore the elucidation of specific protein-protein contacts among polypeptides involved in DNA repair, such as those found by this study, is likely to continue to contribute to our understanding of the repair process. □

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In vivo emergence of HIV-1 variants resistant to multiple protease inhibitors

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INHIBITORS of the human immunodeficiency virus type 1 (HIV-1) protease have entered clinical study as potential therapeutic agents for HIV-1 infection. The clinical efficacy of HIV-1 reverse transcriptase inhibitors has been limited by the emergence of resistant viral variants. Similarly, variants expressing resistance to protease inhibitors have been derived in cell culture^{1–10}. We now report the characterization of resistant variants isolated from patients undergoing therapy with the protease inhibitor MK-639 (formerly designated L-735,524). Five of these variants, isolated from four patients, exhibited cross-resistance to all members of a panel of six structurally diverse protease inhibitors. This suggests that combination therapy with multiple protease inhibitors may not prevent loss of antiviral activity resulting from resistance selection. In addition, previous therapy with one compound may abrogate the benefit of subsequent treatment with a second inhibitor.

MK-639 is a potent and selective inhibitor of the HIV-1 protease^{11,12}. During a clinical study of the compound, virus isolates were regularly obtained from patients and tested for susceptibility in cell culture to a panel of six protease inhibitors.

The inhibitors selected for the panel are compounds or closely related analogues that are either in clinical trials or are being considered for such trials: MK-639, XM323 (refs 13, 14), A-80987 (ref. 15), Ro 31-8959 (ref. 16), VX-478 (ref. 17) and SC-52151 (ref. 18). In addition, viral RNA encoding the protease was amplified by reverse transcription and polymerase chain reaction (RT-PCR), cloned and the DNA sequenced.

After 24 weeks of MK-639 therapy, viruses isolated from Patient A exhibited a fourfold loss of susceptibility to MK-639 (Table 1), as well as resistance to XM323 and A-80987. DNA sequence analysis of multiple independent clones of the variant's protease coding region (Table 2) revealed five to six amino-acid differences from the North American/European clade B consensus sequence¹⁹. This represented seven to eight amino-acid substitutions from the week 0 isolate, three of which, 12(I→T), 16(E→G) and 64(V→I), were reversions to the consensus sequence. The remaining five substitutions, 10(L→R), 46(M→I), 63(L→P), 82(V→T) and 84(I→V), represented forward mutations that appeared during MK-639 therapy.

The genetic basis for the resistance was defined by constructing a series of mutant proviruses expressing various combinations of the observed forward mutations. These were tested for inhibitor susceptibility in cell culture. The results (batch 1; Table 3) showed that coexpression of the three substitutions 46(M→I) + 63(L→P) + 82(V→T) engendered resistance to MK-639, XM323, and A-80987 as seen in the patient A, week-24 virus isolate. The loss of any one or more of these substitutions abolished resistance to MK-639.

By week 40 of therapy, new viral variants that exhibited increased resistance to the inhibitor were present in patient A (Table 1). Notably, these variants expressed a striking cross-resistance to all protease inhibitors tested. The protease coding sequences of the isolates carried numerous additional amino-acid substitutions (Table 2).

The simplest of these patterns, with five amino-acid changes from the consensus sequence, was selected for further study. Varying degrees of resistance were seen, depending on both amino-acid substitution and test inhibitor. Alteration of 84(I→V) alone mediated significant resistance to XM323 (batch 2; Table 4), as previously shown for inhibitor RPI 312 (ref. 2). Also, expression of this substitution in the context of specific changes at positions 10, 46 and 63 produced resistance to Ro 31-8959, VX-478 and SC-52151. However, cross-resistance to all

TABLE 1 Susceptibility of patient viral isolates to multiple protease inhibitors

Patient	Week*	Concentrations (nM) giving 95% inhibition					
		MK-639	XM323	A-80987	Ro 31-8959	VX-478	SC-52151
A	0	100	200	800	25	100	200
A	24	400	1,500	3,000	50	200	200
A	40	1,500	3,000	3,000	800	400	3,000
A	52	>3,000	>3,000	>3,000	800	800	>3,000
B	52	1,500	800	>3,000	200	400	800
C	44	1,500	3,000	>3,000	100	1,500	800
D	44	800	1,500	>3,000	100	1,500	1,500

Values represent 95% inhibitory concentrations for virus replication in human peripheral blood mononuclear cell cultures²², and were determined using serial twofold dilutions of test compounds. Values shown in bold are significantly different (at least fourfold) from those obtained for the week 0 virus isolate. Early isolates from patients B, C and D were fully susceptible and phenotypically identical to those of the patient A, week-0 virus. At least two determinations were obtained for each isolate-inhibitor combination.

* Virus isolates were obtained from patients at the indicated times during therapy with MK-639, and were derived by peripheral blood mononuclear cell co-cultivated as described²².

TABLE 2 Amino-acid substitutions in protease region of viral isolates

Patient	Week*	Amino-acid residue																							
		10 L	12 T	13 I	16 G	20 K	24 L	32 V	35 E	36 M	37 N	41 R	46 M	47 I	54 I	57 R	63 L	64 I	71 A	72 I	82 V	84 I	90 L	92 Q	93 I
A	0	V	I		E										K		V								
	24†	R										I			K	P				T					
	24†	R										I			K	P				T	V				
	40†	R										I				P				T	V				
	40†	V	I				I				D	I				P		V		T	V				
	52	R				I	I					I		V		P		V		T					
B	0							D	I		K						V								
	52	I				M	I	D	I		K			V		P		V		A					
C	0															P, L			V						
	44‡							I				I, L				P	M	V	V, I	A					
D	0†			V								I, M				P, L	I, V								L
	44‡			V				I, V				I	V, I			P	V					M, L	K		L

Amino-acid sequences were deduced from the nucleotide sequences of the protease coding region. Substitutions relative to the consensus sequence (second line of table) are shown (see text). Total RNA was prepared as previously described²³ using 0.2-ml samples of culture medium from infected peripheral blood mononuclear cell cultures. First-strand cDNA was synthesized in 120 µl reaction volume with the oligonucleotide primer d(CTTTGGGCCATCCATTCCTGGC) and Superscript II RNase H⁻ reverse transcriptase (RT) (Gibco-BRL) using conditions specified by the manufacturer. Twelve portions of 10 µl of each RT reaction were independently amplified by nested PCR. The Gag-RT region was first amplified using the above primer and the 5' primer d(CAGAGCCAACAGCCCCAGCAG) with the GeneAmp PCR reagent kit and AmpliTaq DNA polymerase (Perkin Elmer). Conditions were as recommended by the manufacturer, except that amplifications were performed at 1.5 mM MgCl₂ with 0.13 µM TaqStart antibody (Clontech) in a Perkin Elmer PCR9600 thermal cycler for 25 cycles of 94 °C, 15 s; 56 °C, 1 min; 72 °C, 2 min. The protease gene was amplified from portions of 10 µl of the first PCR reaction in 100 µl final volume using primers d(ACGCGUACUAGUGGGACACCATATGGCTACTGTATCCTTTA(G/A)C(T/C)TCCC) and d(AUGGAGAUUCUGTGTGAAGCTTAAGTTCAATAGGACTAAT(G/T/A)GG). Amplification conditions were as described above except that cycling was performed at 94 °C, 15 s; 61 °C, 1 min; 72 °C, 2 min. Each PCR product was gel-purified and cloned into either pAMP19 (Gibco-BRL) or pGEM-T (Promega) using the manufacturer's conditions. The insert from one colony from each PCR reaction was dideoxy sequenced on both strands with dye terminators using Applied Biosystems 373A DNA sequencers. Typically, 8 to 11 independent clones from each viral sample were sequenced, and, except as noted, the reported sequences were present in the majority of clones. Because only one clone from each PCR reaction was sequenced, all clones are assured to be independent. Thus the determined distribution of sequences should be representative of the starting population.

* Virus isolates were obtained from patients at the indicated times during therapy with MK-639, and were derived by peripheral blood mononuclear cell co-cultivation as described²².

† These isolates were mixed cultures, yielding an approximately equal representation of clones expressing the two sets of substitutions noted here.

‡ The mixed amino-acid substitutions noted at positions 32, 46, 47, 63, 64, 72 and 90 appeared to assort independently.

six inhibitors required a minimum of four substitutions in the protease: 46(M→I) + 63(L→P) + 82(V→T) + 84(I→V).

The pattern of mutations present in the patient A, week-52 isolate is more complex. The level of resistance to MK-639 at week 52 had at least doubled from that observed at week 40 (Table 1). Sequence analysis of these viruses (Table 2) revealed that four new amino-acid substitutions had occurred and, surprisingly, the 84(I→V) mutation had been lost from seven of eight clones. None the less, the viral population was still highly cross-resistant to all inhibitors tested (Table 1). Although the 84(I→V) substitution had been pivotal in the development of broad cross-resistance at week 40, its absence at week 52 indicates that alternative pathways to broad-spectrum protease inhibitor resistance exist.

In three other patients treated with MK-639, broad cross-

resistance to protease inhibitors also became apparent after extended therapy (Table 1). Viruses isolated at week 52 from patient B and week 44 from patients C and D showed distinctly different patterns of eight, five and six new amino-acid substitutions, respectively (Table 2). The genetic bases for these alternative pathways of cross-resistance are not yet clear.

These data and sequence analyses of viruses from other patients treated with MK-639 (J.H.C. *et al.*, unpublished observations) suggest that alterations of residues 82(V→A, F or T) or 90(L→M) are necessary but not sufficient for measurable resistance to MK-639. These effects, and those of 32(V→I) and 84(I→V), can be explained by influences on inhibitor binding because their side chains lie in close proximity to the active site^{11,20,21}. The influences of the other substitutions (notably at positions 10, 20, 24, 36, 46, 54, 63 and 71) on resistance, however,

TABLE 3 Susceptibility of constructed mutant viruses to multiple protease inhibitors (batch 1)

Virus*	95% inhibitory concentrations (nM)					
	MK-639	XM323	A-80987	Ro 31-8959	VX-478	SC-52151
NL4-3 (wild type)	100	200	800	25	100	200
10(L→R)	50	200	800	50	50	100
46(M→I)	100	200	800	25	100	200
63(L→P)	50	100	1,500	50	100	200
82(V→T)	100	100	1,500	25	100	100
10(L→R)+46(M→I)	100	200	800	25	50	200
10(L→R)+63(L→P)	50	200	800	25	50	200
46(M→I)+63(L→P)	50	400	800	25	50	200
46(M→I)+82(V→T)	100	400	1,500	25	50	400
63(L→P)+82(V→T)	100	400	1,500	25	100	200
10(L→R)+46(M→I)+63(L→P)	50	200	800	25	200	200
10(L→R)+46(M→I)+82(V→T)	200	200	1,500	25	50	100
10(L→R)+63(L→P)+82(V→T)	200	800	3,000	25	200	400
46(M→I)+63(L→P)+82(V→T)	400	800	3,000	25	100	400
10(L→R)+46(M→I)+63(L→P)+82(V→T)†	400	800	3,000	25	200	400

Mutant viruses were constructed using gapped-duplex oligonucleotide mutagenesis²⁴ of a subclone of plasmid pWT-6 (ref. 25). Infectious mutant proviral clones were reconstructed by subcloning the 833-b.p. *Apal*-*Sse8387I* fragment containing the mutagenized protease gene into the corresponding sites of plasmid pNL4-3 (ref. 26). After transfection of the mutant proviral clone into HeLa cells and growth of viral stocks in cocultivated H9 human T-lymphoid cells²⁷, the complete sequence of the viral protease gene from the mutant viral population was verified as described in the legend for Table 2. Values represent 95% inhibitory concentrations (nM) for virus replication in the MT-4 human T-lymphoid cell line²⁸, using serial twofold dilutions of test compounds. The values shown in bold are significantly different (at least fourfold) from those obtained for the wild-type virus.

* Substitutions are noted by amino-acid residue number in the viral protease.

† This mutant's protease substitutions are identical to the new mutations seen in the patient A, week-24 isolate (see Table 2 and text).

TABLE 4 Susceptibility of constructed mutant viruses to multiple protease inhibitors (batch 2)

Virus	95% inhibitory concentrations (nM)					
	M-639	XM323	A-80987	Ro 31-8959	VX-478	SC-52151
NL4-3 (wild type)	100	200	800	25	100	200
84(I→V)	50	3,000	800	50	100	400
46(M→I)+82(V→T)+84(I→V)	100	1,500	3,000	50	50	800
63(L→P)+82(V→T)+84(I→V)	200	3,000	3,000	50	200	800
10(L→R)+46(M→I)+63(L→P)+84(I→V)	200	3,000	1,500	100	400	1,500
10(L→R)+46(M→I)+82(V→T)+84(I→V)*	—	—	—	—	—	—
10(L→R)+63(L→P)+82(V→T)+84(I→V)	100	3,000	1,500	100	400	800
46(M→I)+63(L→P)+82(V→T)+84(I→V)	800	3,000	3,000	200	800	1,500
10(L→R)+46(M→I)+63(L→P)+82(V→T)+84(I→V)†	800	3,000	3,000	200	800	1,500

See Table 3 for experimental details.

* Viruses derived from two independent constructs of this mutant grew too poorly in cell culture to allow phenotypic analysis. The complete DNA sequence of the mutagenized fragment in the final constructs was verified. Introduction of a 63(L→P) mutation into one of these plasmids restored viability of the derived viruses, indicating that the combination of 10(L→R)+46(M→I)+82(V→T)+84(I→V) mutations is deleterious for virus growth.

† This mutant's protease substitutions are identical to those of one variant seen in the patient A, week-40 isolate (see Table 2 and text).

are more obscure. These substitutions have been repeatedly observed in viral sequences from other patients on MK-639 therapy (J.H.C. *et al.*, unpublished data), strongly suggesting that they confer selective advantages for viral growth. They may act indirectly through long-range structural perturbations of the protease to increase enzymic activity in the presence of the inhibitor. The biochemical basis for their selection, however, remains to be determined.

The efficacy of therapeutic agents for treatment of infections is often abrogated by selection for resistant variants of the pathogen. This has been shown in response to inhibition of the HIV-1 reverse transcriptase. This report provides the first evi-

dence that inhibition of HIV-1 protease can also lead to the emergence of drug-resistant variants *in vivo*. We have also shown that this resistance can extend beyond the selective agent to include at least five other structurally diverse protease inhibitors. These observations reflect the common nature of the inhibitors' target and suggest that combination therapy with multiple protease inhibitors may yield multiply resistant variants. In addition, initial therapy with any one inhibitor may limit the benefit of subsequent treatment with the remaining compounds. The data argue in favour of biochemically divergent, as opposed to convergent, strategies for combination therapy of HIV-1 infection. □

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PDBBrowse — a graphics interface to the Brookhaven Protein Data Bank

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With a few keystrokes and mouse button clicks on a UNIX workstation, the entire Protein Data Bank archive is now available for searching and displaying the three-dimensional protein structures contained within.

THE Brookhaven Protein Data Bank (PDB) has developed an X-windows-based interactive browser for searching PDB's database of three-dimensional (3D) structures of biological macromolecules. It greatly enhances the PDB's printed index listings and various *ad hoc* search protocols that have been developed for finding PDB entries. Its highly modular and flexible design allows for rapid modification to meet changing needs. Multiple search strings covering various search fields, corresponding to the different PDB entry header record types (such as compound, header, author, or biological source), are supported, using boolean 'and', 'or' and 'not' operators. The selected entry files can be retrieved automatically, and the molecular structures can be displayed using the public-domain X-based molecular viewer RasMol (or a similar viewer).

PDB background

The PDB archives the experimental findings describing, to atomic resolution, the 3D structures of more than 3,000 proteins, nucleic acids and other biological macromolecules. Each one of these data entries is an annotated ASCII text file, identified by a unique 4-character ID-CODE. The average size of a typical entry is over 4,000 lines or 300 kb, and the entire database, currently being updated about every three weeks, is doubling in size every two years.

This amount of data is beginning to present a significant problem for those

wishing to search the entire collection. For example, a simple *ad hoc* author search on an MIPS R3000 SGI workstation at 33 MHz, using the UNIX 'head' and 'grep' commands, took 10 minutes of clock time, compared with 12 seconds with the browser. Such a search becomes prohibitively slow if the collection is mounted on multiple CDs. Even searching index listings, both PDB-supplied and those created by its users, which investigators have relied upon until now, is becoming too inefficient and unwieldy. Typically such indices cover only a subset of possible search fields, such as compound and author name, which makes it impractical to search other data fields (for example, by heterogens or experiment type) or combinations of search strings.

To remedy this situation, the PDB now provides a browser utility that allows a complete text search of the PDB entries online in a friendly, X-windows-based environment. All components of this browser have

been written using public domain products, which are freely available to users, as is the source of the browser.

A number of other tools have been developed to aid in searching the PDB. Some of these are commercial products, which have search capabilities not provided by the PDB browser, for example, IDITIS (marketed by Oxford Molecular, Mountain View, California), MacIcmdad (marketed by Molecular Applications Group, Palo Alto, California) and others. In contrast, the

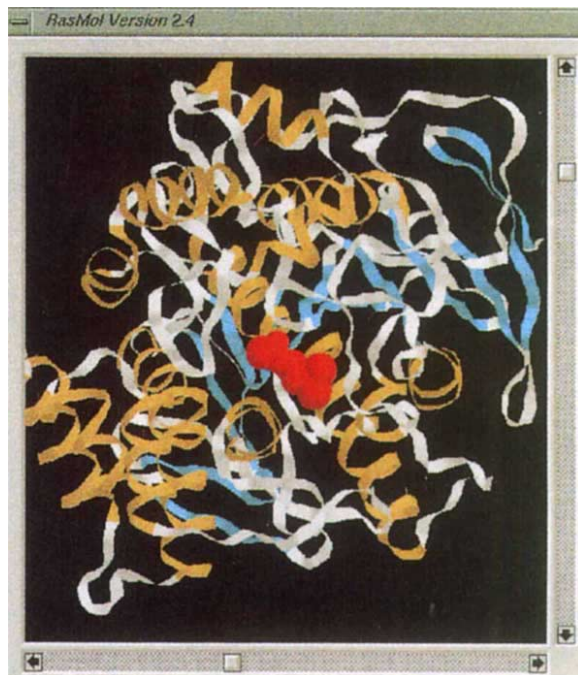


FIG. 2 The PDB browser in action. Above is one of the selected entries, for example, acetylcholinesterase (1ACE) was retrieved into the molecular viewer RasMol. At right is the browse screen, with windows to enter search strings in the appropriate categories. The 'Display Options' add boolean logic to multiple search questions. The 'Search Full PDB' provides an option to search the entire PDB or to just search the list from a previous query.

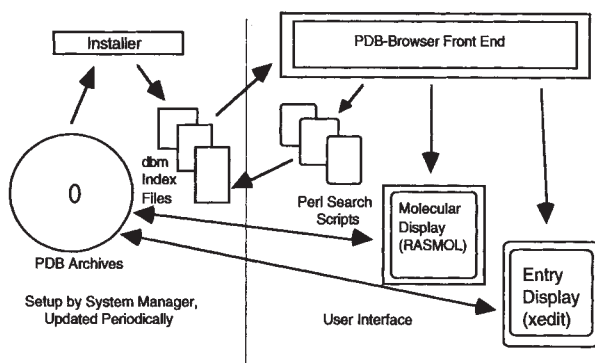


FIG. 1 Modular design of the PDB browser: The index files are written in UNIX hashed dbm database format, and the scripts in Perl³ command interpreter language.

PDBBrowse, as well as a number of search tools developed in other laboratories, are in the public domain. One such utility is SCOP (Structural Classification of Proteins), which has recently been developed by A. Murzin, S. Brenner, T. Hubbard and C. Chothia at the MRC Laboratory of Molecular Biology and Centre for Protein Engineering, Cambridge, UK¹. It provides a detailed and comprehensive description of the structural and evolutionary relationships of proteins whose 3D structures have been determined and is available on the World Wide Web (WWW)², (URL: <http://scop.mrcmb.cam.ac.uk/scop> or <http://ncbi.nlm.nih.gov/repository/scop/index.html>). Although there is some overlap with the PDBBrowse, SCOP is structured completely differently, and thus complements rather than duplicates PDBBrowse.

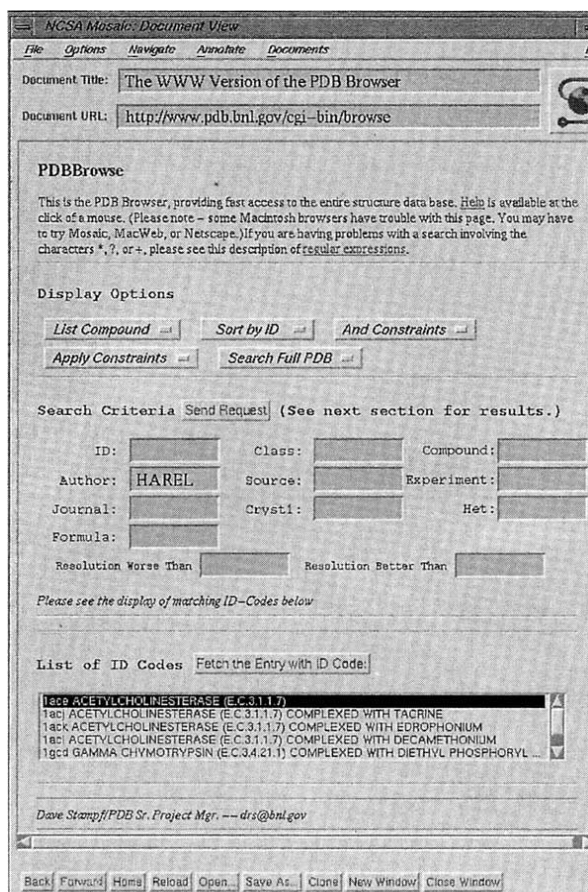


FIG. 3 The PDBBrowse showing the path to find acetylcholinesterase (1ACE)⁷.

Modular architecture

The browser utility, consisting mostly of a series of Perl³ scripts, is constructed in a modular fashion, as shown in Fig. 1. First, the installation Makefile invokes a Perl script to create the different index files, one for each PDB header record type, in UNIX hashed dbm format. Second, to use the browser, one starts up its friendly, window front end, called simply 'browse', one version of which runs under the public-domain command language and windowing management utility 'tcl/tk'⁴. The modular design has made it easy to modify the current tcl/tk front end with an 'html' form in order to support a second front-end, accessible via the WWW (URL: <http://www.pdb.bnl.gov>)⁵. The actual searches are done using a set of Perl scripts called by the front end, that search the index files. (Users without access to an X-windows or WWW interface can run searches by invoking these scripts directly.) The selected entries can then be retrieved and displayed by user-specified utility programs called from the front end.

With the modular design of the browser, each component is independent of the others, so if the format of the PDB collection were to be modified or changed drastically, only the scripts creating the index files would need to be changed.

to the different record types in the header portion of PDB entries. These include, but are not limited to, the chemical compound, the source of the protein, its functional classification, the experimental technique, heterogen molecules present in the structure, author names, crystallographic data, resolution, and remarks. One can combine search strings using .AND. or .OR. logical combinations, using the set of boolean logical switches located below the search fields. Subsequent searches can be performed on the entries selected. (Running a completely new search requires clearing all fields, via the Edit menu.) A online summary of each entry selected by the search is displayed at the bottom, in a format that the user can modify using the 'List Format' menu on the top. The user can then select any of these entries to retrieve and display via the standard X-windows editor 'xedit', (or a different editor selected by the user in environment variable \$EDITOR), and display the 3D molecular picture via the public-domain molecular viewing application 'RasMol'⁶ (or a different molecular display program specified by the user in environment variable \$MOLVIEW).

For particular applications, it is quite easy for the end user to modify the browser, to add custom search scripts and

Additional indices covering new search fields can be added easily at any time, as can new methods for viewing or displaying the retrieved data.

With this arrangement, every time the PDB collection at a given installation is updated, the index files also have to be updated. This can be accomplished quite easily, however, by downloading or installing the updated index files that are provided with the PDB distribution. (Nevertheless, the file location index, which is unique to each site, must be updated locally by typing the command 'hash.pl'.) This capability allows those sites that choose to have their PDB holdings updated automatically over the network, using the 'mirror' application, to have the index files updated automatically as well, and in a completely transparent way.

Usage

As shown in Figs 2 and 3, the top portion of the browser front end allows the user to enter search strings into one or more data fields that correspond

include the corresponding filters and front-end entry windows. For example, a Perl script (simhead.pl) was written that defines a set of equivalence classes on the PDB, based upon the functional classification of the entry. Using this custom script, the browser located all 136 entries with classifications matching those found in the HAREL search illustrated in Fig. 3. The elapsed time for this search was under 4 seconds. One could also envision custom scripts that constrain the search by the volume or shape of the molecule. Everyone is invited to contribute custom scripts to the PDB for possible inclusion in future releases of the browser.

Installation information

The PDB browser may be obtained over the Internet by anonymous ftp to <ftp.pdb.bnl.gov>, gopher to <gopher.pdb.bnl.gov>, or WWW to <http://www.pdb.bnl.gov>. It is also located on the PDB's CD-ROM releases of July 1994 and later. Source codes for the complete browser package, as well as distribution releases for the public-domain products Perl³, tcl/tk⁴ and RasMol⁶, will all be found under directory /pub/pdbbrowse, each in its own separate subdirectory. Before downloading and installing, be sure to examine file 'ReadMeFirst' for complete instructions. For optimal performance, the complete PDB distribution should be mounted locally to the system in use. However, having a remote mount to a disk or CD on another workstation is also quite satisfactory. If neither is possible, the program can access the PDB on another distribution site by using ftp automatically. The time necessary to install the entire package (Perl, Tcl/tk or Mosaic, RasMol and the browser itself) should be under 3 hours. In case of difficulties, you are invited to send electronic mail to the browser's author, David Stampf, stampf@bnl.gov.

Future activities

By the time this article is published, we expect to have released a version of the browser that uses index files at the PDB via a network connection. Thereafter, as updates to the PDB are released by any method of distribution, these index files will also be updated automatically with them.

This browser was inspired by a similar program for MS DOS/MS Windows, called pdbshell, that was written by Enrique Abola and Eugene Ko. That program is based on the Foxpro database program and is also available on the PDB file server and CD-ROM under the directory /pub/pdbshell. A version of that browser for Macintosh is currently under development, as RasMol has recently been released for the Macintosh. □

The PDB is supported by the US National Science Foundation, the US Public Health Service, the US National Institutes of Health

(National Center for Research Resources, National Institute of General Medical Sciences and the National Library of Medicine) and the US Department of Energy and user fees. We wish to thank Enrique Abola and the members of PDB for their help with this work. D.R.S. is in the Department of Chemistry, Brookhaven National Laboratory, Upton, New York 11973 USA. C.E.F. is in the Department of Structural

Biology, Weizmann Institute of Science, Rehovot 76100 Israel. J.L.S. holds a joint appointment with the Department of Structural Biology at the Weizmann Institute of Science and Brookhaven National Laboratory. For more information, fill in reader service number 100.

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Experimental biology

Featured this week in product review are gel analysis systems for molecular biology research, cultureware for neuronal cells, an optical biosensor system and a molecular weight analyser.

INCSTAR has introduced **rabbit antiserum to calcitonin gene-related peptide (CGRP)**, which is useful for immunohistochemical research applications in the human spinal cord as well as that of a number of animal species (*Reader Service No. 101*). Immunohistochemical studies have demonstrated that CGRP is widely distributed in the central and peripheral nervous systems. The antiserum against this neuro-peptide was generated in rabbits to synthetic rat α -CGRP (1-37) and coupled to bovine thyroglobulin with glutaraldehyde. The specificity of the antiserum for immunohistochemistry was examined by soluble pre-adsorption with the peptides in question at the final concentration of 10^{-5} M. The manufacturer states that immuno-labelling was eliminated by pre-adsorption with rat α -CGRP and partially eliminated by pre-adsorption with rat β -CGRP, whereas pre-adsorption with the following peptides resulted in loss of immunostaining: rat amylin, rat adrenomedullin, calcitonin, neurotensin, somatostatin, substance P, leucine enkephalin, methionine enkephaline, VIP, CCK-8, vasopressin and neuropeptide Y.

MaxPlax packaging extract from Epicentre Technologies utilizes a new restriction-free packaging strain to achieve what the manufacturer states are extremely **high packaging efficiencies for λ DNA** ($1-3 \times 10^9$ PFU/ μ g DNA) (*Reader Service No. 102*). This packaging is also designed to enhance packaging of λ -DNA bearing the mammalian methylation pattern. The product is supplied as predisposed single-tube reactions (five or ten extracts per kit). MaxPlax is designed to be an easy-to-use reagent with applications in constructing cDNA libraries, genomic cloning of highly modified DNA into λ -phage or cosmid vectors and rescuing λ -shuttle vectors from transgenic animals.

Fisons Applied Sensor Technology states that epitope mapping of proteins can be dramatically simplified and accelerated



IAsys optical biosensor sensor — Fisons.

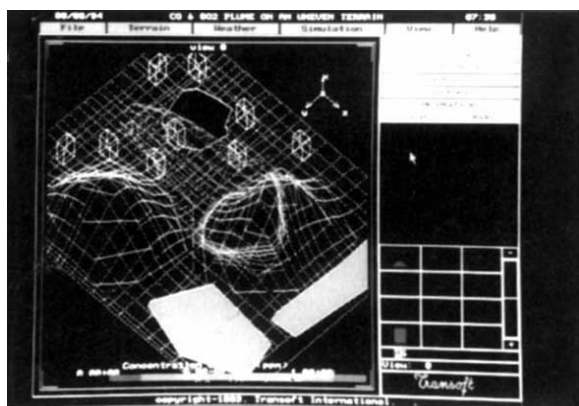
using the **IAsys optical biosensor system** (*Reader Service No. 103*). Such mapping, combined with pre-existing information, can provide insights into the antigen's structure and function. In addition, it allows monoclonal antibodies to be categorized by the epitope they recognize. The system studies interactions between biomolecules using a resonant-mirror sensor, integrated into a 200 μ l stirred cuvette. This obviates the need to purify and label the monoclonal antibodies, or to carry out time-consuming conventional immunoassays. The antigen is immobilized on the IAsys biosensor surface and crude, unlabelled, hybridoma supernatants are added sequentially, followed by the regeneration of the antigen.

A new formulation of [35 S]methionine that can be stored at 4 °C without any affect on performance is now available from Amersham Life Science (*Reader Service No. 104*). Redivue L-[35 S]methionine is designed to be used in all experiments where a standard formulation is used to label proteins by either *in vivo* or *in vitro* methods. [35 S]methionine normally requires storage at -70 °C, but the new Redivue formulation avoids repeated

freeze/thaw cycles and sub-aliquoting. The intense red dye of Redivue products is clearly visible in cell culture media or wheat germ lysate, even at 1 in 50 dilutions, allowing simple determination of which reaction mixes contain the label. Redivue L-[35 S]methionine is available at a specific activity of >37 Tbq mmol^{-1} in a radioactive concentration of 370 Mbq ml^{-1} .

Research Biochemicals carries a range of **research-grade dopamine agonists** (*Reader Service No. 105*). New compounds include Quinelorane, a selective dopamine agonist for the D_2 -like receptor family with a partial ergoline structure, and Quinpirole, which has a greater affinity for D_2 dopamine receptors and greater *in vivo* potency. A frozen aqueous suspension of membranes prepared from CCL1.3 mouse fibroblasts transfected to express the human D_3 dopamine receptor is also available.

Transoft now offers Fluidyn software for **simulating the spread of various types of pollution** from spills, leaks and explosions into the atmosphere, rivers, coastal regions and inland areas so users can then develop an effective plan of action (*Reader Service No. 106*). The software is designed to represent critical



Fluidyn software from Transoft International.

topological and meteorological parameters, allowing users to represent the dispersion of gases and particles over rough terrain and to account for the effects of factors such as buildings, tunnels and cloud cover. The interface allows environmental engineers who are neither programmers nor fluid dynamic specialists to enter data, perform calculations and extract results. Fluidyn runs on standard workstations as well as 486 or Pentium-based personal computers and includes several modes: 'panache' simulates chronic or single incident atmospheric pollution; 'Bruleur' analyses combustion in domestic and industrial burners and furnaces; 'flow coast' predicts the dispersion of waste and spills in rivers lakes, oceans and coastal areas; 'pollusol' analyses contamination of soil, ground water or wells; and 'container' simulates the rupture of reservoirs and pressurized containers by violent shock, impact, fire or explosion.

Collaborative Biomedical Products/Becton Dickinson Labware now offer BioCoat **cellware designed for the culture of a variety of neuronal cells** (*Reader Service No. 107*). The culture surfaces of these tissue culture vessels contain a thin uniform layer of natural or synthetic substrates that are frequently used by neurobiologists: poly-D-lysine, Matrigel matrix, and a mixture of laminin/poly-D-lysine. This product is available as coverslips, Multiwell plates, dishes and flasks — each vessel is available in several sizes, except coverslips. BioCoat thin-layer Matrigel cellware promotes axonal and dendritic outgrowth from primary neurons and neuroblastomas and differentiation of PC12 cells. Poly-D-lysine or poly-L-lysine cellware is useful for culturing dorsal-root ganglia, cortical sympathetic and hippocampal neurons. Poly-D-lysine/laminin cellware promotes attachment of neuronal cells and neurite outgrowth and supports survival of primary neurons and glial cells in culture.

■ Instruments

Shimadzu Scientific has designed Kompact MALDI I, a combined matrix-assisted, laser-desorption ionization, time-of-flight mass measurement system, to be an affordable **MALDI molecular weight analyser** (*Reader Service No. 108*). The instrument is designed primarily for accurate analyses of molecular weight in the biotechnology, pharmaceutical and polymer industries. A range of sample substrates may be selected to provide optimum results from a variety of different sample types, ranging from synthetic industrial polymers to protein enzyme digests. A continuous scan mode provides flexibility for PVDF blots, HPLC and CZE fractions. Windows-based software includes all control, pro-

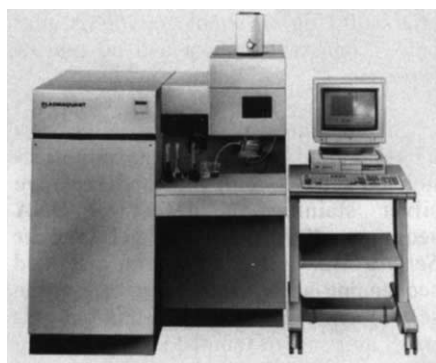


MALDI I molecular weight analyser.

cessing and diagnostic software, as well as packages for peptide/protein analysis, peptide database searching and industrial polymer calculations.

The Anti-HIV-1/HIV-2 EIA DAGS from Roche Diagnostic Systems is the latest addition to the range of assays available on the Cobas Core **assay system** (*Reader Service No. 109*). The new assay is based on the direct double-antigen sandwich (DAGS) principle, which allows the simultaneous detection of IgG, IgM and IgA directed against HIV-1 including subtype O and HIV-2. The manufacturer states that this assay shows sensitivity for HIV-1 and HIV-2 in low-titre and seroconversion panels. The high values for sensitivity and specificity are because of the use of recombinant and synthetic antigens presented in their native conformation. The inclusion of the entire HIV-1 gag24 sequence and the use of natively folded proteins results in detection of HIV-1 subtype O with a total incubation time of about a 120 min.

The Plasmaquant 110 from Carl Zeiss, is a high-resolution **Echell spectrometer** with a fibre-optic light guide system to project each spectral line to its own radiation detector (*Reader Service No. 110*). The spectrometer positively identifies and quantitatively registers 70 elements by means of 132 selected spectral lines covering the wavelength range from 193 nm to 852 nm. Measurements are made simultaneously in 12 channels, with an analysis programme comprising up to 60 lines. A



Plasmaquant 110 from Carl Zeiss.

monochromator mode enables any spectral line, other than the 132 standard lines, to be selected for identifying 'exotic' samples. Measurement and data analysis software supports the compilation of methods and calibration, the elimination of spectral interferences, routine analysis programs and self-diagnostics.

■ Separation techniques

Beckman has introduced the eCAP **chiral methods development kit for capillary electrophoresis** (*Reader Service No. 111*). This kit is designed for rapid development of methods for the separation of optical isomers on the company's P/ACE capillary electrophoresis system. The manufacturer states that capillary electrophoresis is an important tool for chiral separation due to its high efficiency and minimal consumption of solvents, buffers and additives. The eCAP kit is stated to allow optimization in days rather than weeks, with resolution and selectivity maximized in as few as eight runs. It contains two neutral capillaries, β -cyclodextrin, γ -cyclodextrin, hydroxypropyl- β -cyclodextrin, dimethyl- β -cyclodextrin, three buffers, two pH adjusters, test mix, a chiral method development guide and a diskette.

FMC BioProducts has announced the release of MetaPhor XR (*Reader Service No. 112*). This agarose is designed to form flexible **gels for high-resolution separation of small DNA conformers** (<1 kilobase), and should prove useful in mutation detection (heteroduplex and cold PCR-SSCP analysis). The manufacturer states that this agarose is compatible with most horizontal and vertical electrophoretic equipment and combines the convenience and ease of agarose with the resolving power of polyacrylamide. MetaPhor XR agarose is designed for agarose-gel mutation detection.

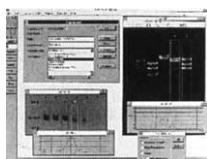
Advanced Biotechnologies now offers high-capacity fast-flow **phosphocellulose gel matrix** (*Reader Service No. 113*). The matrix allows purification of DNA binding proteins including transcription factors, polymerases, kinases and other proteins that have a special affinity for the phosphate-sugar backbone. The gel is ready for use requiring no removal of fines and does not exhibit the clogging tendencies or slow flow rates of conventional phosphocellulose gels.

Gallard-Schlesinger/BDH Laboratory Supplies have announced a new range of high-purity **isoelectric point markers for native-gel isoelectric focusing** (*Reader Service No. 114*). These gels are designed to overcome the confusion of identification caused by stray unaccounted for protein bands in gel separations. The new kits have all the bands calibrated and cover the pH ranges 2.4–6.1,

PRODUCT REVIEW

4.7–9.2 and 7.3–10.6. Coloured isoelectric focusing markers, Nonidet P-40 and other speciality products for electrophoresis are also available.

The Gel-Pro analyser, from Media Cybernetics, is a set of applications designed for molecular biologists involved in **gel analysis and protein research** (Reader Service No. 115). This instrument features automated gel reading functions with programing written with standard molecular biology terms and follows conventional laboratory protocols. It analyses PCR, northern, Southern and western blots as well as restriction length polymorphisms. Included are proprietary algorithms designed to correct for smearing, slanted lines/bands and signal intensity variations. Gel-Pro also provides a wide range of experiment management functions for the storage and retrieval of images.



Gel-Pro analyser.

B/T Scientific Technologies has designed the Speedlight Platinum for low-cost **gel documentation** (Reader Service No. 116). The system comes with a hard-copy output capability and a digital disk storage device for the capture and transfer of TIFFs to floppy disk. It features image saturation detection, time/date/text stamping, 15,000 frames of integration and background subtraction. The floppy drive allows 15-s central capture of images to a 3.5 inch disk, which then can be taken to a convenient computer depending on the users' preferences and design application. The system provides 768 × 472 image capture that is timed by the pixel clock of the charged-coupled device camera, eliminating aliasing and providing high resolution. A cabinet houses the video-imaging system, integration/capture device, monitor, and thermal printer, allowing both benchtop operation (no darkroom needed) and protecting the researcher from ultraviolet irradiation. The operator can preview the image on the active video monitor, eliminating some of the guesswork on camera settings. Macintosh or PC analysis software determines integrated density and molecular weight of selected bands.

■ DNA peripherals

The Centrisart-C4 **microconcentrator**, from Sartorius, has been designed for fast ultra- and microfiltration of small samples (Reader Service No. 117). Applications include concentrating and purifying DNA and proteins; buffer exchange and desalting of samples; removal of low-molecular-weight components, such as PCR primers, non-incorporated nucleotides, peptides and unbound ligands; and

removal of agarose particles from eluted DNA. This microconcentrator has a filter area of 0.28 cm² and a maximum centrifugal force of 10,000g. It consists of a filter insert with a maximum sample capacity of 400 µl, and of a 2.0 ml microcentrifuge tube to collect the filtrate. The membrane of the filter insert is available in a choice of various cutoffs or in a standard pore size and has low nonspecific protein binding. For quick identification, the molecular weight cutoff and membrane type are printed on the filtrate tube. The etched area can be labelled indelibly.

Stratagene's RoboCycler 96 **gradient temperature cycler** features a new higher throughput, 96-well format (Reader Service No. 118). The system uses a robotic arm to move samples from one of four custom-programmed, preset temperature blocks to another, eliminating the need to ramp block temperature. The unit provides a gradient temperature in the second block, which can produce a linear temperature gradient of up to 22 °C across all 12 of its rows. This linear gradient allows testing of multiple primer annealing temperatures in one experiment. After the optimal temperature is determined, the gradient block can also be run in the single-temperature mode. The manufacturer states that even the outer wells of the 96-well blocks show a well-to-well temperature uniformity of ±0.1 °C. The unit accommodates 96-well plates, 200-ml tubes or eight-tube strips and is available both with and without the gradient feature.

DuPont NEN extends the shelf life of **non-radioactive labelling and detection kits** with its new Renaissance products (Reader Service No. 119). The random primer fluorescein dUTP labelling kit and nucleic acid chemiluminescence reagent have a manufacturer-stated shelf life of one year. Non-radioactive labelled probes produced with this kit are said to have a greater stability than traditional ³²P-labelled probes. Detection is stated to be faster using the kits: comparable signal levels have been obtained in less than 5 min using fluorescein-labelled probes compared with radioactively labelled probes exposed for 24 hours. It can detect as little as 0.1 pg of homologous DNA after only 1 min of treatment and 60 min of exposure.

Silver Sequence from Promega combines thermal cycling (linear amplification of a DNA template) and sensitive silver staining to **detect a DNA sequence directly in the gel** (Reader Service No. 120). It uses a patented sequencing-grade *Taq* polymerase as the sequencing enzyme. The system requires small amounts of template (8–16 ng of a 200 base pair PCR product and 2–4 µg of a 3,000–5,000 bp supercoiled plasmid)

and can be used to sequence PCR products directly without the need for subcloning. The kit contains all the staining and sequencing reagents necessary to go from template to readable sequence in a day. □

These notes are compiled by Brendan Horton from information provided by the manufacturers. For more details, fill in the reader service card bound inside the journal.

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Reader Service No. 11

Nature's sister journals in review

Strategies to protect against or attack HIV, and the latest on breast and ovarian cancers, feature in the April issues of *Nature Medicine*, *Nature Structural Biology* and *Nature Genetics*.

Poxvirus as a vector for vaccination against HIV

As well as triggering the generation of antibodies, a successful vaccination strategy should induce cytotoxic T-cell production. Conventional vaccination with antigen/adjuvant complexes is not always very efficient at this, and a number of other systems are being explored, including the use of recombinant attenuated poxviruses. On page 321 of the April issue of *Nature Medicine*, A. B. Abimiku *et al.* show how effective such a strategy can be: four out of eight rhesus macaques immunized with recombinant poxviruses expressing HIV-1 subunits were resistant to subsequent infection with HIV-2, indicating that the variability of HIV-1 need not necessarily preclude the eventual development of a successful vaccine for humans.

Two different strains of poxvirus were used, one based on vaccinia, but lacking 18 genes implicated in viral virulence, and the other based on a poxvirus from canaries, which is unable to replicate in non-avian cells. Both, however, retain the powerful ability of poxviruses to induce cytotoxic T-cell production. Various combinations of HIV genes were inserted into each of these viruses, and each was used to immunize two rhesus macaques. Each member of a pair was then given a different combination of HIV subunits as a booster inoculation, so that no two macaques received the same treatment.

nature
medicine

Rhesus macaques are not susceptible to HIV-1, so it was only possible to evaluate their response in terms of the levels of specific antibodies and cytotoxic T cells. They are, however, susceptible to HIV-2, allowing Abimiku *et al.* to test how far the immunized macaques were protected against this related virus. Although the difference between HIV-1 and -2 is much greater than that between any two strains of either virus, three out of the eight vaccinated macaques were nonetheless completely protected against a subsequent challenge with HIV-2, and onset of disease was significantly delayed in a fourth. All three control animals and the four remaining vaccine recipients showed strong viral replication.

None of the antibody titres tested in the vaccinated animals were clearly correlated with immunity, nor were the levels of cytotoxic T cells. Moreover, the authors caution that the small number of animals

used and the variety of treatment protocols employed preclude drawing statistically significant conclusions from the data. Nevertheless, poxvirus vectors clearly offer a promising route to the broad-based humoral and cellular immune response that will be necessary if humans are ever to be protected against so variable a pathogen as HIV.

Later in infection, though, augmented cellular immunity can be too much of a good thing. On page 330, S. Koenig *et al.* discuss the effects of infusing an expanded autologous cytotoxic T-cell clone specific for the nef protein of HIV-1 into an HIV-1-positive volunteer. So far from reducing viral load and inducing clinical remission, the patient's CD4⁺ T-cell count fell after both infusions, accompanied by clinical deterioration and a rise in viral load. In the most recent samples from the patient, 20 per cent of the virus had deletions in the epitope recognized by the T-cell clone. Nevertheless, the presence of large numbers of T cells recognizing an epitope present in most circulating virus is clearly no bar to ongoing viral replication and pathogenesis. □

Perturbations of *BRCA1* in sporadic cancers

HOPES that the identification, six months ago, of the *BRCA1* gene linked to familial breast and ovarian cancer would quickly lead to an understanding of what happens in sporadic tumours appear to have foundered, but in a puzzling fashion. That comes on top of the disappointment that familial breast and ovarian cancer is associated with not just one or a few mutations of *BRCA1* but with at least 38 mutations, conveniently summarized in a News and Views article by J. Boyd (page 335 of this month's *Nature Genetics*).

A search for mutations in sporadic cases of breast cancer proved negative, but now S. D. Merajver and colleagues (page 439) have found four somatic mutations in 47 examples of apparently sporadic ovarian cancer. (They also identified five germline mutations among the sporadic ovarian cancer examples, four of which are identical and which are also present in 5 per cent of normal chromosomes, not associated with ovarian cancer.) On page 343 of the same issue,

L. Hosking and colleagues describe their finding of one *BRCA1* mutation in an apparently sporadic case of ovarian cancer.

In another approach to understanding the role of *BRCA1* (page 444), M. E.

Thompson *et al.* have examined the activity of *BRCA1*. Their essential finding is that *BRCA1* is more actively expressed in normal than in cancerous breast tissue, and that an antisense

nature
genetics

Nature Medicine: other points

■ The A1 allele of the D₂ dopamine receptor has been associated with a predisposition to alcoholism, and D₂ receptor agonists seem to be effective in treating the condition. But are their effects more pronounced in individuals with the A1 allele? A double-blind study of 83 patients suggests the answer is yes, with A1 homo- and heterozygotes showing a reduction in craving for alcohol, and less depression and anxiety.

■ The genes responsible for two forms of hereditary colon cancer are known, and kindreds carrying dangerous alleles can be screened for early signs of cancer. Now a study of patients without a family history shows that 18 out of 31 patients under 35 carry mutations predisposing to replication errors, so screening of unusually young patients may also be justified.

Nature Genetics: other points

■ The role of trinucleotide repeats in genetic disease may be explained by a postulated DNA structure of successive base-triplets.

■ A transgenic mouse model of cystic fibrosis is susceptible to lung pathogens associated with the human disease.

■ A study of DNA from an eight-year-old's skull at Tel Akhziv (Israel) reveals β -thalassaemia compensated for by overproduction of fetal haemoglobin. The skull dates from the 16th–19th centuries.

oligonucleotide directed against the site of the *BRCA1* gene product accelerated the growth of cultured breast cancer cells in a dose-dependent manner.

The last finding is taken as confirmation that *BRCA1* is normally an influence against the proliferation of mammary epithelial cells, both in normal and cancerous tissues. Although somatic mutations have not been shown in breast tumours, Thompson *et al.* suggest that *BRCA1* may play a role in sporadic breast cancer if its expression is insufficient to prevent the proliferation of cells, because of either faulty regulation or splicing. (There are 24 exons in the gene.)

A further puzzle raised by these reports is that occasioned by the observation of loss of heterozygosity (LOH) in the *BRCA1* region in breast tumours even when there is no sign of somatic mutations. The LOH phenomenon, a further sign that *BRCA1* is a tumour-suppressing gene, presumably arises (as in other

tumours) by recombination and natural selection.

The association of somatic mutations of *BRCA1* with ovarian but not mammary tumours leads Boyd to raise the question of whether the gene may have been misnamed for 'breast cancer'. He also suggests that it may be difficult to match the 'spectacular' history of the gene with therapeutic benefit.

Finally (page 401), multiplication ('amplification') of the androgen receptor gene on the X chromosome appears to be responsible for the emergence of resistance to anti-androgen therapy in roughly 30 per cent of cases, according to a Finnish group (T. Visakorpi *et al.*). The authors propose that the explanation may be the selective advantage of amplification in the presence of low concentrations of nontesticular androgens, suggesting clinical trials of procedures for removing residual androgens in patients with recurring prostate cancer. □

Views of HIV proteins for the drug designers

Two papers in this month's *Nature Structural Biology* tackle the problem of mobility in the HIV-1 protease — a target for anti-AIDS therapeutics, also discussed on pages 494 and 569 of this issue of *Nature* — using two different approaches. One approach is theoretical (J. R. Collins *et al.*, page 334), the other experimental (L. K. Nicholson *et al.*, page 274).

Proteins are constantly in motion and these motions are often crucial to function. This is particularly true of the HIV-1 protease, whose active site is guarded by two flaps which must open in order to allow substrates, and drugs designed to inhibit the protein, to enter the site.

Despite the large number of structures of the protein (at least 200) the details of flap-opening are unclear and previous studies have failed to simulate the process. Collins *et al.* succeeded by forcing the structure from an inhibitor-bound to an unliganded form. This initial impulse was sufficient to stimulate flap-opening, thus allowing the identification of conformational changes in four amino acids at the base of the flaps as responsible for the bulk motion of the flaps.

Experimentally, nuclear magnetic resonance (NMR) spectroscopy is extremely powerful in the study of protein motions as the individual mobility of almost all atoms in the protein can be measured. Nicholson *et al.* have placed HIV-1 protease under this intense scrutiny. They find that the flaps are undergoing large-scale motions on a fast (picosecond to nanosecond) time scale and, moreover, that the tips of the flaps are undergoing a

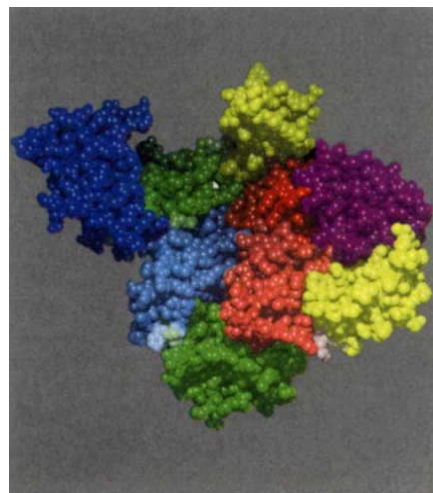
conformational change on a slower (microsecond) time scale.

These two studies complement each other perfectly, in that they pinpoint the same amino acids as important for flap opening. They also bear on drug design by showing how some drug-resistant mutants evade inhibition by changing the dynamics of the flaps. Indeed the flaps of one such mutant could not be opened by Collins *et al.*, presumably preventing drug binding.

Drug-based treatments of AIDS can be likened to putting a molecular spanner in the works of the virus. All the currently approved anti-AIDS drugs — AZT, ddI and ddC — are substrate (nucleoside)-analogue inhibitors of the HIV reverse transcriptase (RT) enzyme. These drugs directly block the active site, thereby interfering with the conversion of viral RNA into proviral DNA and hampering viral replication. A second, chemically diverse, class of inhibitors are the non-nucleoside inhibitors (NNIs) which interfere only indirectly with the reactions at the active site. In two other papers in the April issue of *Nature Structural Biology*, J. Ren *et al.* (page 293) and R. Esnouf *et al.* (page 303) now present the first high-resolution structures of the complete HIV-1 RT, both alone and in complex with four different NNIs. The drug/RT complexes reveal a common molecular interface between the different NNIs and provide pointers as to how drug designers might tackle the problem of drug resistance in an attempt to develop more effective treatments for AIDS.

The structure of the RT heterodimer (formed from p66 and p51 subunits) has

been likened to a hand, which can grip the cylindrical double-stranded nucleic acid; thus the different domains have names like 'fingers', 'palm' and 'thumb'. The active site of the enzyme is in the



Structure of HIV-1 RT, coloured to show its domain structure. The drug nevirapine is just visible in pale grey (top centre).

palm domain. All four of the NNIs studied are found to occupy a hydrophobic pocket — also in the palm domain — some 10 Å from the polymerase active site. A common feature of the different NNI/RT complexes is the extensive hydrophobic interactions between the aromatic rings of the NNIs and the residues lining the cavity.

The drugs seem to insinuate themselves between two complementary hydrophobic surfaces in the pocket; indeed, the effectiveness of an inhibitor seems to depend to a large extent on how well it mimics the contours of these complementary surfaces. Prizing apart the two surfaces substantially distorts the conformation of the active site, inactivating the enzyme. And it is the structural plasticity of the surrounding protein that accommodates the different chemical substituents of the four NNIs.

Although NNIs are very potent RT inhibitors, their use results in the rapid appearance of resistant viruses — both *in vitro* and in clinical trials — which has limited their use as a stand-alone treatment of AIDS (their potential as part of multi-drug combination therapy is still being explored however). The structures reveal that resistant mutants for the most part reduce the specific hydrophobic interactions between the pocket residues and inhibitors. The observation that there are no known resistance mutations for at least a couple of critical residues which contact the NNIs — implying that these residues have important functional roles in RT — will provide some encouragement for drug designers. Construction of compounds that use these residues to interact with, and inhibit, the enzyme may be less susceptible to the development of resistant strains of HIV. □